Action of surfactants on Saccharomyces cerevisiae as influenced by lipid composition.

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ACTION OF SURFACTANTS ON *SACCHAROMYCES CEREVISIAE* AS INFLUENCED BY LIPID COMPOSITION

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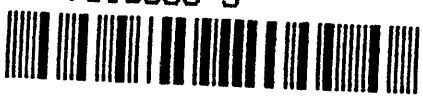

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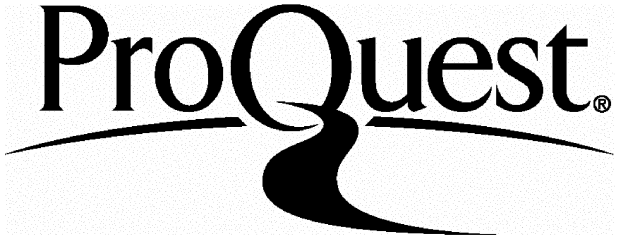

ABBREVIATIONS

The following abbreviations are used throughout this thesis:

CTAB.....Cetyltrimethylammonium bromide

PC.....Phosphatidylcholine

PE.....Phosphatidylethanolamine

SDS.....Sodium dodecyl sulphate

All temperatures recorded in this thesis are in degrees Celsius. The abbreviations for the other chemical compounds and units, and the abbreviations of the names of the journals referred to in the bibliography are those recommended by the Biochemical Journal (1978). *169*, 1-27.

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An investigation was made into the effect of enrichment of the plasma membrane of *Saccharomyces cerevisiae* NCYC 366, with either phosphatidylcholine or phosphatidylethanolamine, on the susceptibility to surfactants. The three surfactants used were cetyltrimethylammonium bromide, sodium dodecyl sulphate and Triton X-100.

There was no difference in the loss of viability or release of cations between populations of the two types of enriched cells when suspended in buffered cetyltrimethylammonium bromide. However, populations of cells with plasma membranes enriched in phosphatidylethanolamine lost viability and cations at a greater rate when suspended in buffered sodium dodecyl sulphate. Although Triton X-100 possessed little antimicrobial activity, it was able to induce a similar release of cations from populations of cells enriched in either phosphatidylcholine or phosphatidylethanolamine suspended in buffered 1.2 M-sorbitol. No difference was found in the ability of cetyltrimethylammonium bromide or Triton X-100 to induce release of cations, in buffered 1.2M-sorbitol from sphaeroplasts prepared from cells enriched in either phospholipid, but sphaeroplasts from cells enriched in phosphatidylcholine released cations faster when suspended in buffered sorbitol containing sodium dodecyl sulphate. Liposomes containing potassium chloride prepared from mixtures of phospholipids extracted from cells enriched in either phosphatidylcholine or phosphatidylethanolamine lost potassium ions at the same rate when challenged with either sodium dodecyl sulphate or Triton X-100 but no release

of potassium ions was detected when they were challenged with cetyltrimethylammonium bromide.

Cells and sphaeroplasts enriched in phosphatidylcholine or phosphatidylethanolamine did not differ in appearance in scanning or transmission electron micrographs. There were no differences between the two types of enriched cells, in electrophoretic mobilities over a pH range 2 to 9, in ease with which they were converted into sphaeroplasts with β-glucanase, or in the permeability to a range of probing molecules. Walls from phosphatidylcholine- and phosphatidylethanolamine- enriched cells had similar contents of β-glucans, α-mannan and protein. A time-course study was made of sphaeroplast formation, using cells that were not enriched in either phosphatidylcholine or phosphatidylethanolamine, employing the electron microscope.

The mode of action of the three surfactants is discussed, in particular the possible reasons for the different sensitivities of cells and sphaeroplasts enriched with either phosphatidylcholine or phosphatidylethanolamine to sodium dodecyl sulphate. There is also a discussion on some aspects of the cell wall and sphaeroplasts as revealed by electron microscopy.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

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INTRODUCTION

INTRODUCTION

YEAST

Yeasts have long been an ally of man; indeed as long ago as neolithic times yeast was used to leaven bread. Moreover, the same biochemical process that was used to make bread rise opened mankind to a new world of enchantment. At the beginning of recorded history, beer was being brewed in Egypt and Mesopotamia and by 3000 BC potable alcoholic beverages had become necessities to most societies in Europe and the Near East. Over the next 5000 years, man's manipulation and dependence on yeast increased, culminating in the vast alcohol and baking industries which are of considerable economic importance to modern civilisation. However, the same organism which spawned these industries can also be said to be responsible for the birth of biochemistry.

In the latter half of the 19th Century the stage was set for the emergence of biochemistry with the controversy that raged between proponents of the vitalistic theory of fermentation championed by Cagniard-Latour, Schwann and Kützing, and the chemical theory of fermentation, supported by Leibig, Wöhler and Berzelius. This controversy has since been well documented by both Conant (1952) and Rose (1977a), as have the revolutionary discoveries of Pasteur (1866, 1876) by Vallery-Radot (1902) and Dubos (1950). Following these revelations, one question remained unanswered, namely the exact cause of decomposition of the sugar molecule. This however was answered by the Buchner brothers and Hahn in 1897. By grinding yeast and obtaining a cell-free extract that fermented sugar they showed that fermentation results from the action of substances contained within the cell, thus delivering biochemistry into the age of scientific enlightment.

In the three or four decades that followed this demonstration of cell-free fermentation the first biochemical pathway involving the catabolism of glucose into ethanol and carbon dioxide was elucidated. It is therefore not surprising to find that, with such a long alliance with man, yeast is still used extensively in biochemical and molecular biological research.

Saccharomyces cerevisiae, being a unicellular organism, is relatively easy to manipulate in the laboratory, yet the results of such manipulation may have direct relevance to the cells of higher organisms due to the eukaryotic nature of the yeast cell. Throughout the present investigation, the yeast *Sacch. cerevisiae* NCYC 366 was used. In a composition-function study of this kind it is an advantage to have easy access to the membrane, and be able to discount any interference from the cell wall. Since it is possible to convert cells of this strain into sphaeroplasts within one hour, it was an obvious choice for this investigation. However, *Sacch. cerevisiae* NCYC 366 is not without its disadvantages as, like most brewery strains, it is genetically rather intractable.

The present investigation shows how the lipid composition of the plasma membrane in *Sacch. cerevisiae* affects its sensitivity to surface-active agents. The study exploits the discovery (Hossack *et al.*, 1977) that proportions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the plasma membrane of *Sacch. cerevisiae* can be increased by growing the organism in defined media supplemented with choline or ethanolamine. It was not only hoped to gain an insight into the action of surface-active agents, but also the fundamental importance of phospholipids to membrane structure and physiology.

SURFACE-ACTIVE AGENTS

Surface-active agents have played a major part in man's fight against disease and their use as antiseptics can be traced from the beginning of medical history to the present day. Their cleansing and antiseptic properties have been known since the early days of the Roman Empire; a number of surface-active compounds which occur in plant and animal tissues, such as bile acids, lecithins, glucosides and unsaturated fatty acids have been used for centuries.

Since the beginning of the century, the demand for surface-active substances as wetting agents and emulsifiers has increased, giving rise to major chemical industries. It is not surprising that, with such a diversity of surfactants some were found to be antimicrobial. Domagk (1935) aroused interest in the possible germicidal potential of a whole range of synthetic surface-active substances, which resulted in numerous reports of their antimicrobial properties. Even more recently in the search for antibiotics, a new group of surface-active bactericides have been discovered namely the polypeptide antibiotics. Many surface-active compounds are used as general purpose germicides and antiseptics on the skin and mucous membranes whilst others, such as the antibiotic polymyxin, have been used successfully for the treatment of intestinal infections.

The field of chemotherapy is not the only field in which the surface-active agents are important. They are extensively used in biochemistry especially in the solubilization of biomembranes (Helenius and Simons, 1975) and the characterization of membrane components (Tanford and Reynolds, 1976). Surfactants thus provide a convenient means of separating membrane proteins.

Chemical Structure and Physical Properties

Surface-active agents can be defined as substances which alter the free energy relationships at interfaces upon absorption, that is, the lowering of surface or interfacial tension. Compounds which display such surface-active properties are characterized by a structural balance between one or more water-soluble (hydrophilic or polar) and one or more fat-soluble (hydrophobic or non-polar) groups. Depending on the nature of the charge or the absence of ionization of the hydrophilic group, the surfactant may be classified as anionic, cationic, non-ionic or ampholytic.

Anionic compounds contain a hydrophobic residue which is usually a paraffinic chain or an alkyl-substituted benzene or naphthalene ring, balanced by a negatively charged hydrophilic group which may be a carboxyl, sulphate, sulphonate or a phosphate group.

Cationic compounds have the same type of hydrophobic groups as anionic compounds, but have a positively charged hydrophilic group which may be a quaternary ammonium, sulphonium, arsonium, phosphonium or iodonium group. The surface-active polypeptide antibiotics can be included in this class since they are all basic peptides which are positively charged at neutral pH values.

The non-ionic surface-active agents possess no ionized groups, the hydrophobic portion being balanced by such non-polar groups as polymerized ethylene oxide or polyhydric alcohols, e.g. long-chain polyglycol ethers and alkyl aryl polyglycol ethers.

Lastly, the ampholytic surface-active agents are compounds of mixed anionic and cationic character, being essentially zwitterions e.g. long-chain

amino acids.

Figures 1a and b show the chemical structure of representatives of these four types; for more information the reader is referred to the comprehensive works of Schwartz and Perry (1949) and Schwartz *et al.* (1958).

A unique physical property of surface-active agents is the ability to form micelles. When small quantities of an amphiphile are added to water, part of it dissolves and part forms a monolayer at the air/water interface. The molecules in the monolayer are in equilibrium with monomers in the bulk solution, and each monomer concentration corresponds to a characteristic surface tension. When the monomer concentration reaches a critical value, addition of further amphiphile leads to the formation of micelles. Micelles can therefore be defined as thermodynamically stable colloid aggregates spontaneously formed by amphiphiles above a narrow concentration range (the critical micellar concentration or C.M.C.) at temperatures above the critical micellar temperature.

Antimicrobial Effect

While all surface-active agents which are highly microbicidal markedly lower the surface tension, not all compounds that lower the surface tension of solutions kill micro-organisms. This is aptly demonstrated by the non-ionic group of compounds, which are not microbicidal, nor do they affect microbial metabolism (Baker *et al.*, 1941b, Hotchkiss, 1946). As long ago as 1941 (Baker *et al.*, 1941a) it was established that surface-active agents possess antimicrobial activity depending on their type.

FIG. 1a SURFACE-ACTIVE AGENTS

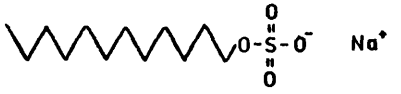
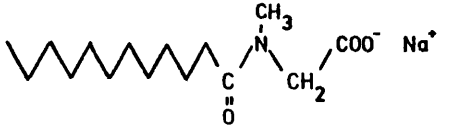
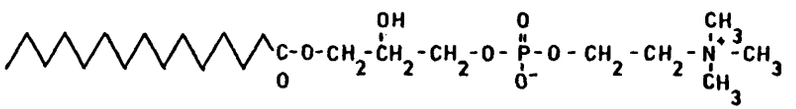
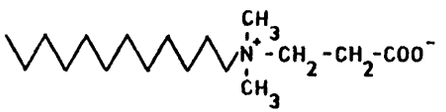
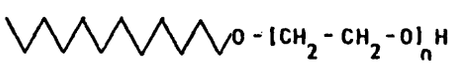
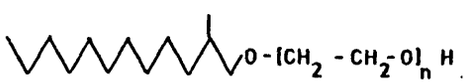
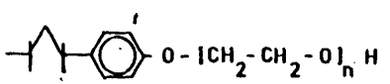
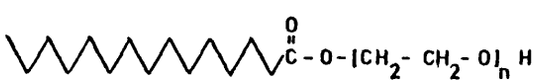
Structural formula	Chemical name
	Anionic surfactants
	Sodium dodecylsulphate
	Sodium dodecylsulphonate
	Sodium dodecyl-N-sarcosinate
	Cationic surfactants
	Cetyltrimethylammonium bromide
	Tetradecylammonium bromide
	Dodecyclpyrimidinium chloride

FIG. 1b SURFACE-ACTIVE AGENTS

Structural formula	Chemical name
	Ampholytic surfactants
	Palmitoyllysolecithin
	Dodecyl-N-betaine
	Non-ionic surfactants
	Polyoxyethylene alcohol
	Polyoxyethylene isoalcohol
	Polyoxyethylene p-t-octyl phenol
	Polyoxyethylene esters of fatty acids

n = average number of ethylene oxide units/molecule

By far the most microbicidal, and thus the most widely used, are the cationic surface-active agents. Since most surfaces possess a net negative charge these compounds are readily absorbed onto surfaces such as glass, wool, proteins and micro-organisms, and are therefore effective against a wide spectrum of organisms. They are almost equally effective against Gram-positive and Gram-negative organisms with maximum effect in alkaline solutions. In contrast, the anionic surface-active agents, while containing several microbicides rivalling the best cationics, generally have a narrower antimicrobial spectrum. They are selectively active against Gram-positive organisms with maximum activity in acid solutions. As already mentioned, non-ionic detergents are not generally germicidal while,with the ampholytic detergents, the bactericidal activity and the pH optimum for activity increase with the number of nitrogen atoms in the molecule.

Anionic, cationic and ampholytic agents all precipitate proteins from solutions which decreases their antimicrobial activity due to formation of surfactant-protein complexes (Glassman, 1949). Anti-microbial activity is also decreased by the presence of phospholipids, possibly due to adsorption of phospholipid onto the microbial surface which subsequently interacts with the surfactant rendering it inactive (Baker *et al.*, 1941b). Mixtures of anionic and cationic surface-active agents will also interact and cause loss of antibacterial properties.

However, it is possible to obtain, with the complementary types of surface-active agents, a synergistic effect. Mixing quaternary ammonium and non-ionic surfactants potentiates their antibacterial properties. Similarly, low concentrations of an anionic surfactant increase the activity of phenol due to an increased penetration rate, but high

concentrations cause a decrease in the activity due to removal of phenol in the surfactant micelles (Moore and Hardwick, 1956).

Molecular Basis of Antimicrobial Activity

That some surface-active agents prevent growth or kill micro-organisms has never been disputed, but in the past where their site of action lies, has led to much controversy. Hugo (1965) grouped the theories of antimicrobial activity into five broad categories, namely effects on proteins, effects on metabolic reactions, the nature of the stimulated glycolysis reaction, the theory of an enzymatically maintained dynamic membrane, and effects on cell permeability.

Effects on Proteins. Putnam (1948) reviewed the action of surface-active agents on proteins and showed that they were potent denaturing agents, while Khun and Dann (1940) demonstrated the disruption of the link between protein and prosthetic groups of enzymes caused by this group of compounds. Furthermore, Gilby and Few (1960) postulated that the site of action of anionic surfactants was protein. However, although both cationic and anionic surface-active agents denature membrane proteins, they do so at much higher concentrations than those which are lethal to microbial cells. It is therefore unlikely that this phenomenon is the primary cause of antimicrobial activity.

Effects on Metabolic Reactions. A large amount of work using a variety of techniques has been published, attempting to correlate antimicrobial activity of surface-active agents to effects on the metabolic reactions. Miller *et al.* (1939) were among the first to show that very low concentrations of an alkyldimethylbenzylammonium chloride inhibited the

respiration and glycolysis of organisms that cause lesions in human teeth. These workers extended (Baker *et al.*, 1941a and b) their study to anionic, cationic and non-ionic surface-active compounds, finding that depression of metabolism roughly paralleled killing and that certain surfactants (especially those in the anionic group) stimulated bacterial metabolism at sub-inhibitory concentrations. Krebs (1948), studying decarboxylation of glutamic acid and glutamine by *Clostridium welchii*, noted that CTAB accelerated decarboxylation of these compounds, whereas SDS inhibited the reaction. Krebs (1948) suggested that this increase in decarboxylation was due to an increased permeability of the cells. However, Hughes (1949) disproved this by finding that CTAB also increased the rate of decarboxylation by cell-free extracts. Armstrong (1957) who studied the effect of six cationic agents on baker's yeast concluded that the initial toxic reaction was disorganisation of the cell membrane and this was followed by inactivation of cellular enzymes. He also noted that low concentrations of surfactant caused small increases in acid production. These, and the other large number of publications on this subject, show that any measure of agreement or disagreement may be demonstrated depending on the enzyme and the test organism.

Stimulated Glycolysis Reaction. It has already been noted that low concentrations of surfactants stimulated glycolysis. This prompted Scharff and Beck (1959) to investigate in detail stimulation of glycolysis by benzalkonium chloride in yeast. The mechanism was finally elucidated by Bihler *et al.* (1961), when they showed that it was due to prevention of further oxidation of acetate. Whilst of interest, these effects occur at lower concentrations than are microbicidal, and therefore cannot be considered significant in antimicrobial action.

Enzymatically Maintained Membrane Theory. It might be expected that an enzyme's cellular location would affect its response to surface-active agents. Thus, an enzyme situated at the cytoplasmic membrane would be affected first. In the light of evidence for the dynamic cell membrane, Newton (1958) postulated this may be maintained by an enzyme which could well be sensitive to some surface-active agents. However, since its proposal there has been no evidence to support this theory, nor is there likely to be in the light of current knowledge of cell membranes.

Effects on Cell Permeability. Haemolysis can be induced by surface-active agents, and it has been extensively studied (Salton, 1968). Micro-organisms, however, unlike mammalian cells possess a cell wall which is responsible for the rigidity of the cell. The outer wall therefore acts as a protective corset preventing breakdown and osmotic explosion of the more plastic protoplast when the cell encounters an environment of low tonicity (Salton, 1964). Removal of the wall by enzyme treatment leaves a protoplast or sphaeroplast, depending on the terminology used. This structure is osmotically sensitive and exhibits a sensitivity to surfactants similar to an erythrocyte. Protoplasts of *Bacillus megaterium* exhibit marked susceptibility to lysis with SDS (Salton, 1957). Gilby and Few (1957) also demonstrated lysis of *Micrococcus lysodeikticus* protoplasts by SDS, sodium dodecyl sulphonate and certain cationic compounds. Further evidence for the primary effect of surface-active compounds on membranes came from Salton (1968) who found that isolated membrane preparations were rapidly dissociated by C_{10}, C_{12} and C_{14} alcohol sulphonates. Indeed, the greater effectiveness of C_{12} and C_{14} alcohol sulphonates correlated with their biological activity on bacteria and erythrocytes.

Membrane damage caused by surface-active compounds however, can also be detected in whole organisms. One of the first indications of this is the release of metabolic pool constituents, such as purine and pyrimidine nucleotides, amino acids and inorganic phosphate (Hotchkiss, 1946; Gale and Taylor, 1947; Salton, 1951). Surfactant-induced membrane damage also permits entrance of certain substances which are normally unable to penetrate the cell (Newton, 1958). Since the evidence that a surface-active agent's prime effect is to disorganize the plasma membrane, it would be expected that its interactions with lipids would play an important part.

Cholesterol has been implicated as the site of action of certain anionic surfactants. However, with the exception of mycoplasmas, bacteria do not incorporate sterols into their membranes, so that cholesterol could not be the site of action in most bacteria . Pethica and Anderson (1953), in monolayer penetration studies, demonstrated that surface-active compounds could penetrate into the phospholipid layers of the bacterial membrane. Phospholipids are known to inhibit the action of surfactants (Baker *et al.*, 1941b). Kondo and Tomizawa (1966) showed that interactions with phospholipids can also play an important role in cell lysis. Dunnick and O'Leary (1970) examined polymyxin-sensitive and resistant strains of *Escherichia coli*, and found that the resistant strains contained a lower proportion of cyclopropane fatty-acyl residues. Polymyxin action was again investigated by Feingold and his co-workers (Hsu Chen and Feingold, 1973; Feingold *et al.*, 1974) who found that liposomes prepared from phosphatidyl-ethanolamine were more sensitive than those prepared from N-methyl-substituted analogues of phosphatidylcholine. From this they

concluded that phosphatidylethanolamine was the target molecule and proposed that the first stage of membrane damage was proton transfer between the antibiotic and the membrane.

Thus there is a strong body of evidence which suggests that surface-active agents cause death of micro-organisms by disrupting the selectively permeable plasma membrane which leads to loss of essential cofactors and metabolites. Since any disruption of the plasma membrane requires penetration by surface-active agents, the lipid composition of the membrane would appear to be a very important factor in such action.

MEMBRANES

Function

A membrane may be defined as a selectively permeable barrier surrounding a cell or organelle. Most prokaryotic organisms possess only one membrane, namely the plasma membrane, which may be invaginated. Eukaryotic organisms on the other hand, possess in addition a complex system of intracellular membranes. The three principal functions of the yeast plasma membrane are firstly, to protect and maintain the internal environment of the cell irrespective of environmental changes; secondly to control the flow of nutrients in and waste products out; and thirdly to be involved with, or to provide a site for, certain enzymes, such as ATPase associated with active transport and those required for wall synthesis (Matile *et al.*, 1969; Hunter and Rose, 1971).

Molecular Architecture

In 1899, from permeability studies on plant tissue, Overton suggested that membranes contained a fatty substance (Overton, 1899). Subsequently analysis has shown membranes are composed mainly of lipid and protein. The year 1925 saw the first of the structural membrane theories when Gorter and Grendel (1925) reported that when lipids, extracted from erythrocyte membranes, were spread as a monolayer at an air-water interface, they occupied an area corresponding to approximately twice the surface of the erythrocyte. This result suggested that the membrane consisted of a bilayer, with the hydrocarbon chains occupying the centre and the polar head-groups facing outwards. Following this discovery, independently, Davson and Danielli (1935) proposed the presence of a lipid bilayer in membranes and, in order to explain anomalously low surface tensions of biological membranes compared with model lipid

systems, they postulated that the lipid core of natural membranes was sandwiched between two layers of protein. Although this theory was based on a misconception, in that phospholipids alone can produce the low surface tensions shown by natural membranes, the Davson-Danielli model dominated membrane biology for over thirty years. The widespread occurrence of apparently similar membranes led Robertson (1959) to promote the concept of the 'unit membrane' based on the Davson-Danielli model. This Davson-Danielli-Robertson model was backed by a large amount of evidence from electron microscope and X-ray diffraction studies.

Inconsistencies in the 'unit membrane' theory led to the 'mosaic model' being proposed by Lenard and Singer (1966) and by Wallach and Zahler (1966). This model was further elaborated by Singer and Nicolson (1972) when they stressed the dynamic aspects of the membrane structure in describing the 'fluid mosaic model'. This is now a widely accepted theory and envisages globular proteins embedded in, and even crossing, a lipid bilayer core. Their now classic diagram shows the globular proteins (icebergs) floating in a lipid bilayer (sea). Evidence to support this theory has come from freeze-fracture electron micrographs, in which protein can be apparently seen in the bilayer (Branton, 1969; Pinto da Silva and Branton, 1970; Marchesi *et al.*, 1972). Furthermore, structural studies on proteins such as cytochrome $\underline{b}_5$ (Ito and Sato, 1968; Spatz and Strittmatter, 1971) indicate that they are amphipathic, and would be likely to have their hydrophilic end protruding and their hydrophobic end embedded in the bilayer (Singer and Nicolson, 1972).

Although the Singer and Nicolson (1972) diagram shows intrinsic proteins (proteins embedded in the bilayer), other proteins extrinsic to the membrane (proteins which exist outside the bilayer and interact

electrostatically with phospholipid, head-groups) are thought to exist. It should be stressed that sterols, which are known to be present in the plasma membrane of *Sacch. cerevisiae,* are not depicted in the Singer and Nicolson diagram. An aspect no diagram can convey is that of movement. However it is now apparent that both proteins and lipids possess the ability to move in the plane of the membrane and possibly across it. Through spin-labelling studies, phospholipids have been shown to exchange places with their neighbouring phospholipids at a rate of 10^7 times per second (Devaux and McConnell, 1972). It has also been shown that phospholipids are also able to 'flip-flop' across the bilayer, but at a rate 10^{10} times slower than neighbour-neighbour exchange (Kornberg and McConnell, 1971).

The fatty-acyl chains of phospholipids are highly mobile at physiological temperatures. At lower temperatures, they are tightly packed in a hexagonal array. As the temperature is increased so the molecular motion of the fatty-acyl chains gradually increases until a sharp rise in heat absorption occurs and the mobility of the fatty-acyl chains abruptly increases giving rise to the fluid or liquid-crystalline state. The temperature at which the gel-to-liquid phase change occurs is known as the phase transition or the order-disorder transition. The transition temperature is affected by the degree of unsaturation and the length of the fatty-acyl chains (Ladbrooke and Chapman, 1969). The composition of the head-group is also known to influence the transition temperature. For instance, the transition of dipalmitoylphosphatidyl-ethanolamine is $20^{\circ}C$ higher than the transition of the homologous choline-derived lipid (Ladbrooke and Chapman, 1969). It is also known that sterols, such as cholesterol, can form equimolar complexes with

phospholipids and lower mobility of the fatty-acyl chains. The study of gel-to-liquid phase changes in biomembranes has not been as successful as those carried out in artificial membranes. The reason for this is that phase changes are not clear cut or even detectable due to the complex nature of biological membranes.

The fluidity of biological membranes does not appear to be a casual feature. Probably all micro-organisms possess a mechanism to regulate the unsaturation of the fatty-acyl residues it incorporates into its membrane, so as to maintain the membrane at least partly fluid at a particular temperature of growth (Sinensky, 1971; Hunter and Rose, 1972). It has been shown that newly synthesised M protein of *E. coli* can only be incorporated into membranes *in vivo* when the lipid component is at least partly fluid (Tsukagoshi and Fox, 1973). The lactose transport system also appears to be dependent on the order-disorder state of the membrane (Cronan and Gelmann, 1975). Thus the maintenance of the plasma membrane in at least a partly fluid state appears essential for its functioning.

From work on the H-2 antigen in the membranes of mouse and human cell heterokaryons, Frye and Edidin (1970) were the first to demonstrate that proteins are able to move in the plane of the individual membrane monolayers. Subsequently, this phenomenon has been observed with many surface antigens and receptors in a wide variety of cells (Singer, 1974). However it should be remembered that the mobility of proteins will ultimately depend on the fluidity of the membrane lipids.

Evidence is now available indicating an asymmetric distribution of lipids and proteins across the two monolayers of plasma membranes, and

also for ordered complexes of lipid-protein in the bilayer. Asymmetric distribution of proteins has been reported in the membranes of a variety of cells including erythrocytes (Nicolson and Singer, 1971), *Micrococcus lysodeikticus* (Salton *et al.*, 1972; Oppenheim and Salton, 1973) and *Candida utilis* (Marriott, 1977). Using lactoperoxidase-catalysed iodination, Marriott (1977) found that the specific activity of labelled membrane of intact protoplasts from *C. utilis* was very low, indicating that very few proteins were present on the outside surface of the membrane. When both surfaces of disrupted protoplast membranes were labelled the specific activity of labelling increased 100-fold, indicating an asymmetric distribution of proteins. However, freeze-etching studies of the plasma membrane of *Sacch. cerevisiae* have revealed a predominance of particles on the outside face (Moor and Mühlethaler, 1963; Takeo *et al.*, 1976). Although these particles have been claimed to contain mannan and protein in the ratio of 5:2 (Matile *et al.*, 1967), all other plasma membranes studied contained the majority of protein, on the inside face. It is therefore unlikely that the particles observed in *Sacch. cerevisiae* account for the majority of the membrane protein.

Lipid asymmetry in erythrocyte membranes was first postulated by Bretscher (1972) and has been subsequently supported by Zwaal *et al.* (1973) and Verkleij *et al.* (1973). Other membranes reported to show lipid asymmetry are milk fat globules (Patton and Keenan, 1975) and influenza virions (Tsia and Lenard, 1975). In these membranes phosphatidylethanolamine and phosphatidylserine predominate in the inside monolayer, facing the cytoplasm, while phosphatidylcholine and sphingomyelin are concentrated on the outer part of the bilayer. Further evidence for asymmetric distribution has come from studies of ionic

drugs on the structure of erythrocytes (Sheetz and Singer, 1974). Anionic compounds were found to cause evagination or crenation and cationic compounds to cause invagination or cups. This apparently is due to the preferential insertion of these compounds into either the inner or outer part of the bilayer. Thus these workers imagined the lipid bilayer to behave as a bimetallic strip and so coined the term 'bilayer couple'. Evidence for asymmetry of lipids is also backed by the work of Wisnieski *et al.* (1974) who were able to detect four phase changes in the physical properties of mammalian cell membranes. They claimed that the two temperatures (15^{o} and 31^{o}C) at which phase changes occur correspond to changes in the outer monolayer of the membrane, whereas those at 21^{o}C and 37^{o}C are due to changes in the inner monolayer of the membrane.

While lipid fluidity and protein mobility appear to be widespread and characteristic properties of membranes, there are instances where membrane components are clearly constrained from being freely mobile in the plane of the membrane. Specialized structures within certain plasma membranes show a high degree of internal order, such as synapses (Whittaker, 1969), gap junctions (Revel and Karnovsky, 1967; Goodenough and Revel, 1970) and the plaques of halobacteria (Blaurock and Stoeckenius, 1971). It is not unreasonable, therefore to suppose that in the plasma membrane of *Sacch. cerevisiae* there are ordered complexes of protein trapping lipid between them. Such specialized complexes would most likely be concerned with functions such as transport and cell-wall synthesis.

Composition of Biomembranes

Investigations of the chemical composition of biomembranes isolated from various sources including animal cells, fungi and bacteria have established a great deal of similarity in overall composition. Biomembranes generally contain about 20 to 40% lipid, 50 to 75% protein and relatively small amounts of polysaccharide. Although proteins are a major component, they have been relatively ignored in comparison with the investigation of lipids.

Membranes usually contain two or three types of phospholipid which account for the bulk of their phospholipid content. However phosphatidylcholine which is common in higher organisms is comparatively rare in bacteria where phosphatidylethanolamine is the major phospholipid (Hilderbrand and Law, 1964; Kates, 1966). Bacteria and the blue-green algae also differ in lacking sterols (Wright, 1961). The mycoplasmas, although unable to synthesize cholesterol, can incorporate sterols (Razin *et al.*, 1963). The presence of sterols in mycoplasma membranes has been correlated with the sensitivity of these organisms to polyene antibiotics and conversely their absence from bacteria and blue-green algae can adequately explain their resistance to polyenes (Lampen, 1966). Absence of sterols from bacterial membranes, however, appears to have no immediate bearing on the sensitivity or resistance of bacteria to anionic or cationic surface-active agents (Salton, 1968).

YEAST PLASMA-MEMBRANE COMPOSITION

Proteins

The protein content of isolated plasma membranes of *Sacch. cerevisiae* has been reported to range from 26% dry weight (Matile *et al.*, 1967) to 65% dry weight (Christensen and Cirillo, 1972). However, the majority of reports have estimated the protein content to be between 35 and 49% dry weight (Boulton, 1965; Suomalainen *et al.*, 1967; Longley *et al* . , 1968; Schibeci *et al.*, 1973). Although they are the major components of the plasma membrane, relatively little is known about them. Three types of protein appear to be associated with the plasma membrane namely, proteins concerned with transport of solutes into the cell, proteins involved with enzymatic functions, and lastly structural proteins.

The existence of transport proteins in the plasma membrane of *Sacch. cerevisiae* is inferred on general physiological grounds. However, there are few reports directly demonstrating the existence of these proteins in those membranes. Fuhrmann *et al.* (1976), using vesicles prepared from isolated plasma membranes of *Sacch. cerevisiae*, have shown that these vesicles exhibit counter transport with glucose and mannose, and iso-counter transport with glucose. From this observation they suggested that a sugar transport carrier exists in the plasma membrane. These workers further showed that the uptake of galactose by vesicles, prepared from galactose pathway induced cells, was 100% more than those of non-induced cells. This increase was explained by the formation of a galactose carrier in the plasma membrane, which was most likely a protein. Other evidence for the existence of transport proteins has come from Patching and Rose (1971), who showed that there was a diminished

rate of accumulation of the non-metabolizable solutes, glucosamine hydrochloride and 2-aminoisobutyrate, after applying cold osmotic shock to *Sacch. cerevisiae*. However, these workers were unable to prove conclusively that proteins involved in transport of these solutes were released from the yeast.

Evidence for the association of enzymes with the plasma membrane of *Sacch. cerevisiae* has come from the work of Christensen and Cirillo (1972), who used plasma membrane vesicles, and Santos *et al.* (1978) who used isolated plasma membranes. Both groups of workers were able to detect invertase and Santos *et al.* (1978) were also able to detect mannosyl transferase activity.

The structural protein tubulin has been isolated from homogenates of yeast (Water and Kleinsmith, 1976). Subsequently Santos *et al.* (1978) have shown the presence of colchicine-binding proteins in the plasma membrane of *Sacch. cerevisiae*, which indicates the presence of tubulin-like proteins. These workers postulated a cytoskeleton exists in the plasma membrane, possibly composed of microtubulules, but more evidence of this is awaited.

Lipids

About 40% of the dry weight of the plasma membrane is composed of lipid (Boulton, 1965; Matile *et al.*, 1967; Suomalainen *et al.*, 1967; Longley *et al.*, 1968); however Schibeci *et al.* (1973) estimated it to be as low as 12.3%. Lipids are water-insoluble organic compounds extractable by non-polar solvents, such as chloroform, ether and benzene. Lipids were amongst the first natural products examined in detail

(Chevreul, 1823), but even so it is only relatively recently with the advent of thin-layer chromatography, that the routine separation of individual lipids from complex mixtures in biological membranes has been possible.

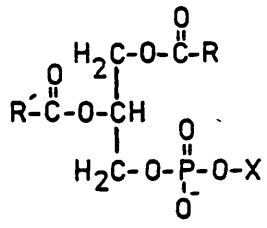
The lipid composition of the plasma membrane of *Sacch. cerevisiae* has been reported by several workers (Boulton, 1965; Matile *et al.*, 1967; Suomalainen *et al.*, 1967; Longley *et al.*, 1968; Christensen and Cirillo, 1972; Schibeci *et al.*, 1973; Kramer *et al.*, 1978). However, the data are very difficult to compare firstly because of the different methods used to isolate the plasma membranes, secondly because of the wide variety of analytical techniques employed and lastly because some groups of workers do not report detailed analysis of lipid fractions. Therefore, in discussing the lipid composition of the plasma membrane, data on polar lipids extracted from cells will also be included as these lipids will almost certainly be membrane components.

The lipid classes found in yeasts are those typically found in eukaryotic organisms; they include glycerophospholipids , sterols, sterol esters, triacylglycerols and glycolipids (Erwin, 1973). However, since only polar lipids can be incorporated into membranes, this review will be confined to glycerophospholipids, sterols and glycolipids.

Glycerophospholipids. Phospholipids are fatty-acyl diesters of *sn* glycero-3-phosphoric acids substituted with any one of several hydrophilic residues such as choline, ethanolamine and inositol (Fig. 2.). There are

two types of structural variation in these molecules centering around the nature of the fatty-acyl and hydrophilic group attached to the phosphodiester bond. Isolated plasma membranes contain about 6% (dry weight) phospholipid (Longley *et al.*, 1968; Schibeci *et al.*, 1973). Although Kramer *et al.* (1978) estimated the content of phospholipid to be about 2.5% dry weight of the plasma membrane, the validity of these workers' results is questionable since they also report that over 90% of the lipid content of the plasma membrane consists of neutral lipids. Most species of yeast have been found to contain 3 to 7% based on the cell dry weight (Letters, 1968a).

The major phospholipids found in the plasma membrane of *Sacch. cerevisiae* are phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol. Longley *et al.* (1968) found that phosphatidylethanolamine accounted for 35%, phosphatidylcholine for 23% and phosphatidylinositol plus phosphatidylserine for 28% of the total phospholipid. However, Kramer *et al.* (1978) found that the amounts of these phospholipids, as a percentage of the total phospholipid, were 20, 34 and 28% respectively. Longley *et al.* (1968) reported similar proportions of phospholipid in extracts from whole cells as they found in the plasma membranes. Other workers have reported percentages of the total phospholipid content for extracts of cells varying between, 25 and 50% for phosphatidylcholine (Jollow *et al.*, 1968; Letters, 1968a; Getz *et al.*, 1970; Hunter and Rose, 1972), and 20 and 32% for phosphatidylethanolamine (Getz *et al.*, 1970; Hunter and Rose, 1972). Phosphatidylinositol is reported to account for about 20% of the total phospholipid extracted from *Sacch. cerevisiae* (Deierkauf and Booij, 1968; Getz *et al.*, 1970; Hunter and Rose, 1972).



R, R´ = Fatty acyl residues

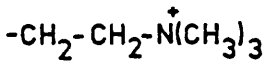
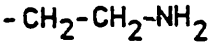
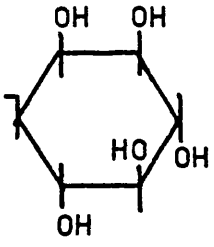
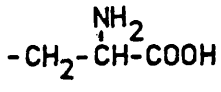
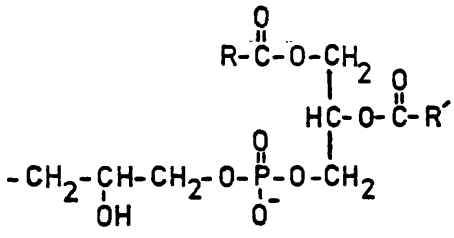
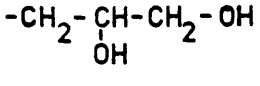
Structure of X	Name of Phospholipid
	Phosphatidylcholine
	Phosphatidylethanolamine
	Phosphatidyl-myo-inositol
	Phosphatidylserine
	Diphosphatidylglycerol (Cardiolipin) R, R´ = Fatty acyl residues
	Phosphatidylglycerol

Fig. 2 Some glycerophospholipids of yeasts

Other phospholipids present in small amounts include phosphatidylserine and diphosphatidylglycerol (cardiolipin). Both of these components individually represent about 4% of the total phospholipid content extracted from *Sacch. cerevisiae*.

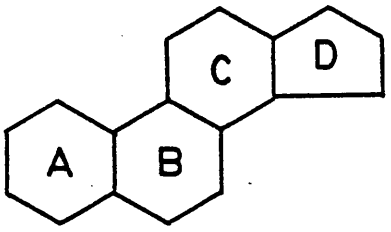
It is widely accepted that the total cellular lipids of *Sacch. cerevisiae* are rich in $C_{16:1}$ and $C_{18:1}$ fatty-acyl residues (Trevelyan, 1966; Longley *et al.*, 1968; Hunter and Rose, 1972). Longley *et al.*, (1968) also reported on the fatty-acyl composition of individual phospholipid classes. They observed an enrichment trend in that $C_{18:1}$ residues were marginally concentrated in the major plasma membrane phospholipids as compared with whole cell phospholipids. Correspondingly $C_{16:1}$ residues were marginally more prevalent in phospholipids not located in the plasma membrane. Of the plasma membrane phospholipids phosphatidylcholine was richest in $C_{16:1}$ residues and phosphatidylinositol plus phosphatidylserine (these phospholipids were not separated) poorest. On the other hand, plasma membrane phosphatidylethanolamine was richest in $C_{18:1}$ residues and plasma membrane phosphatidylinositol plus phosphatidylserine had the highest proportion of $C_{16:0}$ residues.

Sterols (Fig. 3) are based structurally on the cyclopentanoperhydrophenanthrene ring (Fieser and Fieser, 1959; Shoppee, 1964; Klyne, 1965). The sterol content of plasma membranes of *Sacch. cerevisiae* was found to be 6% of the dry weight of the membrane by both Longley *et al.* (1968) and Schebeci *et al.* (1973). The total sterol content of yeasts, including free and esterified forms usually comprises 0.1 to 2.0% of the cell dry weight (Bills *et al.*, 1930; Shaw and Jefferies, 1953), although Dulaney *et al.* (1954) did report 7 to 10% for a particular

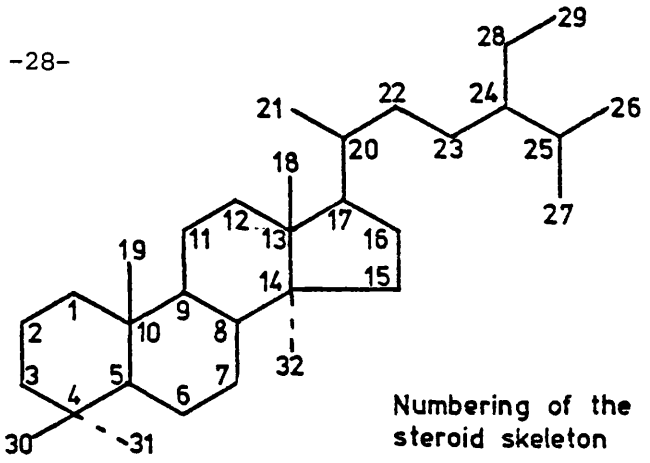
strain of *Sacch. cerevisiae* under certain growth conditions. Ergosterol is the principal sterol found in most yeasts (Wieland and Benand, 1942; Usden and Burrell, 1952; Dulaney *et al.*, 1954; Hunter and Rose, 1971), which is usually followed by 24 (28)-dehydroergosterol, although Longley *et al.* (1968) found that they were present in equal amounts in *Sacch. cerevisiae* NCYC 366. Zymosterol has also been reported in many yeasts (Dulaney *et al.*, 1954) and it has been proposed as an intermediate in ergosterol biosynthesis (Fryberg *et al.*, 1973).

It can be safely predicted that not all of the sterol in a cell occurs in membranes; this is because a large proportion of cellular sterol in *Sacch. cerevisiae* is esterified at C-3 with a long fatty-acyl chain. Sterol esters are not amphipathic and cannot therefore be incorporated into membranes, but they are thought to have a function in the biosynthesis of membranes.

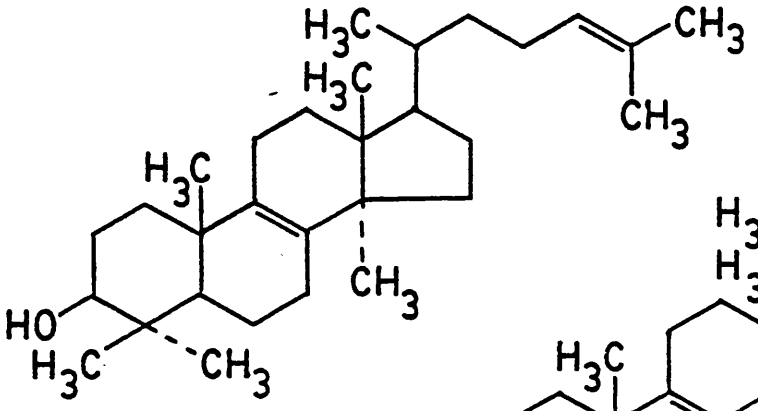
Glycolipids have a head-group attached via the glycoside linkage of a sugar molecule to a diglyceride residue. Small quantities of glycolipids have been isolated from cells of *Sacch. cerevisiae* (Bergelson *et al.*, 1966; Baraud *et al.*, 1970; Nurminen and Suomalainen, 1971; Työfinoja *et al.*, 1974). They are characterized by a relative abundance of long chain fatty-acyl residues (longer than C_{18}). Because of their extremely amphipathic character, it is almost certain that most of the glycolipids extracted from yeasts are located in membranes. Indeed, the presence of steryl glycosides, sulpholipids and acyl glucoses has been observed in the cell envelope of *Sacch. cerevisiae* (Työfinoja *et al.*, 1974). Steryl glycosides appear to be restricted to the plasma membrane whereas monogalactosyl diglycerides and sulpholipids are distributed



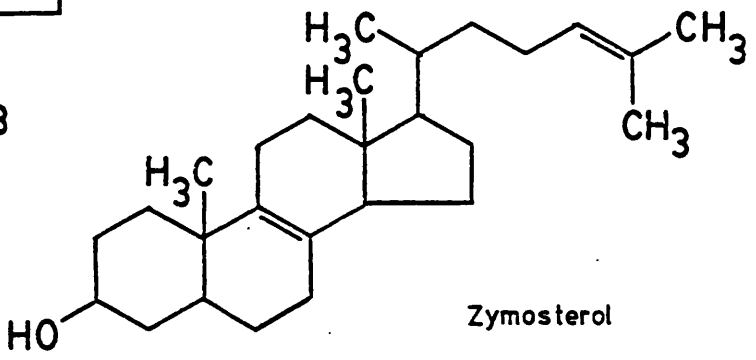
Cyclopentanoperhydro-phenanthrene



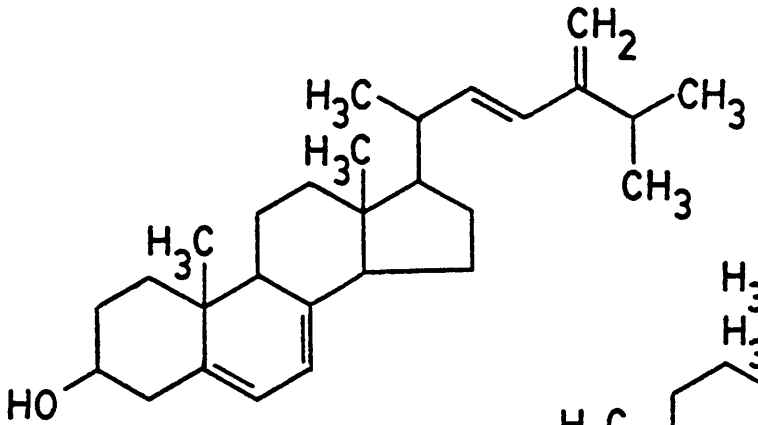
Numbering of the steroid skeleton



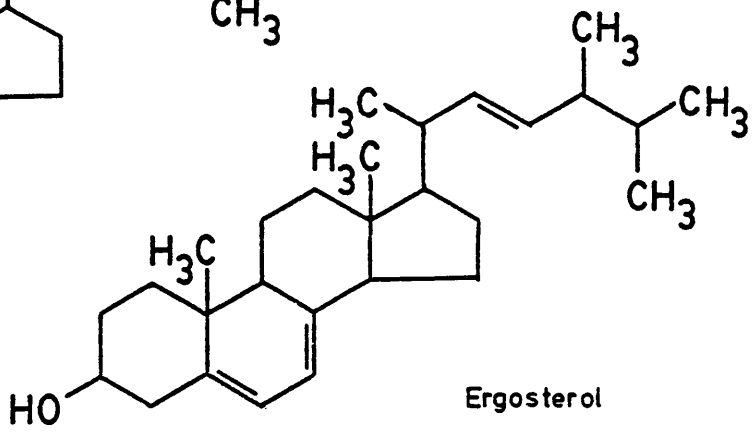
Lanosterol



Zymosterol



24(28)-Dehydro-ergosterol



Ergosterol

Fig. 3 Some important sterols of yeasts

throughout the cell.

Minor components. There are a number of minor phospholipids that have been detected, these include various lysoderivatives, phosphatidylglycerol, phosphatidylglycerol phosphate, phosphatidic acid, phosphatidylmonomethylaminoethanol, phosphatidyldimethylaminoethanol, diphosphoinositol and triphosphoinositol (Getz *et al.*, 1970; Jakovcic *et al.*, 1971; Steiner and Lester, 1972). It now appears that the relatively high proportions of lysophospholipids (Jakovcic *et al.*, 1971) may be artifacts due to the action of phospholipases during extraction (Letters, 1968a).

Other minor components include glycosylated and non-glycosylated sphingolipids. For a more detailed review of lipids discussed here, and other minor components, the reader is referred to the accounts of Hunter and Rose (1971) and Rattray *et al.* (1975).

STRATEGEMS FOR CHANGING THE COMPOSITION OF MEMBRANES

It is well known that the lipid composition of micro-organisms may be affected by a number of factors and this, therefore, calls for strict control of conditions applied during growth of cultures. However, this phenomenon can be used to alter deliberately the composition of membranes, such that hopefully a particular lipid is replaced by stoicheiometric amounts of another lipid without the chemical structure of any other lipid or protein being changed. The experimenter, once he has achieved this highly desirable situation, is then in a position to discover how these compositional changes affect one or more functions of the organelle.

One of the easiest and best documented ways for changing the lipid composition of organisms is to alter the physical or chemical properties of the environment (Hunter and Rose, 1971; Rattray *et al.*, 1975; Rose, 1977b). The most intensively studied environmental factors are growth temperature (Hunter and Rose, 1972) and oxygen tension of the culture (Jollow *et al.*, 1968). Unfortunately both of these factors have multiple effects on the lipid composition of *Sacch. cerevisiae*, thus making composition-function studies difficult.

Four principal ways have been established for effecting specific alteration to the membrane lipid composition of *Sacch. cerevisiae* using nutritional means. The first of these exploits the anaerobically-induced requirement of *Sacch. cerevisiae* for sterol and unsaturated fatty acid. The second involves growing *Sacch. cerevisiae* in the presence of ethanolamine or choline which leads to synthesis of increased proportions of phosphatidylethanolamine and phosphatidylcholine respectively. The third is the

use of lipid requiring mutants. Finally the lipid composition of *Sacch. cerevisiae* can be altered by growth in the presence of certain drugs. Since the second of these strategems forms the basis for this study it will be considered in more detail in a later section.

Over 20 years ago Andreasen and Stier (1953, 1954) found that *Sacch. cerevisiae* grown under strictly anaerobic conditions becomes auxotrophic for sterol and unsaturated fatty acid, because molecular oxygen is required for synthesis of these compounds. Fortunately the requirements for both sterol (Proudlock *et al.*, 1968) and fatty acid (Light *et al.*, 1962) are fairly non-specific, thus providing an excellent means for effecting changes in the sterol and unsaturated fatty-acyl composition without causing any alteration in the amounts of the total lipid synthesized or in the nature and proportions of the individual phospholipids (Hossack and Rose, 1976).

By selecting for a characteristic after mutagen treatment, a mutant can be produced deficient in certain biochemical functions. Through investigation of this mutant an insight can be gained into the functioning and effect of a specific gene. However their use in manipulating the composition of the plasma membrane of *Sacch. cerevisiae* has not been extensive. Two main classes of mutants in haploid strains of this yeast have been described, namely those which have a mutation in genes that control the synthesis of, and therefore require fatty acids, and those that are unable to synthesize sterols. Mutants unable to synthesize unsaturated fatty acids were first described by Resnick and Mortimer (1966). Since they require oleic acid for growth they are commonly referred to as the 'ole' mutants. A second series of fatty acid-requiring mutants

are the chain elongation mutants and these have a requirement for saturated fatty acid (Schweizer and Bolling, 1970; Schweizer *et al.*, 1971; Henry and Keith, 1971). Mutants that require sterol for growth have proved much more difficult to isolate because of the existence in *Sacch. cerevisiae* of several alternative pathways between lanosterol and ergosterol. However Karst and Lacroute (1974) were able to isolate a mutant of *Sacch. cerevisiae* which required ergosterol for growth. So far, fatty acid- and sterol-requiring have not been used extensively for altering the composition of the plasma membrane of *Sacch. cerevisiae*. Their main use, and this applies particularly to the 'ole' mutants, has been in research on the biogenesis and function of mitochondria (Linnane and Crowfoot, 1975).

A potentially useful, but as yet not fully exploited, strategem for effecting specific changes in the lipid composition of the plasma membrane of *Sacch. cerevisiae* is to grow the organism in the presence of drugs which inhibit specific reactions that lead to synthesis of individual lipids or lipid classes. Sterol synthesis can be inhibited in *Sacch. cerevisiae* using a drug (SKF 4401-A). The same organism grown in the presence of the glycerol analogue 3-chloropropane 1,2 diol has shown a decreased content of phosphatidylglycerol (Bulman and Stretton, 1975). Another potentially useful drug is cerulenin, which inhibits the biosynthesis of fatty acids and sterols in *Sacch. cerevisiae* (Nomura *et al.*, 1972a, b). Inhibition can be reversed by adding exogenous fatty acid, although the organism is still able to extend the chain length and modify the unsaturation of the fatty acid (Ōmura, 1976).

ENRICHMENT WITH PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE

Lester and his colleagues (Waechter *et al.*, 1969; Waechter and Lester, 1971) discovered that by including a low concentration (1 mM) of choline chloride in a chemically defined medium, *Sacch. cerevisiae* can be induced to synthesize a greater proportion of phosphatidylcholine. Subsequently, Ratcliffe *et al.* (1973) showed that including the same concentration of ethanolamine in a medium induced a proportionally increased synthesis of phosphatidylethanolamine by *Sacch. cerevisiae* NCYC 366. It has since been shown that the plasma membranes are also significantly enriched in phosphatidylcholine and phosphatidylethanolamine respectively (Hossack *et al.*, 1977) as shown in Table 1. It can be seen that the proportions of other phospholipids are little affected except for phosphatidic acid in the plasma membrane of choline-enriched cells. This unusually high content may be due to the action of phospholipases on phospholipids.

There are two separate pathways leading to synthesis of phosphatidylethanolamine and phosphatidylcholine. These are the methylation pathway and the cytidine nucleotide pathways. The reactions that take place on the methylation pathway are shown in Fig. 4; these involve synthesis of phosphatidylserine from CDP-diglyceride,decarboxylation of phosphatidylserine to yield phosphatidylethanolamine and the stepwise methylation of this intermediate to produce phosphatidylcholine. Steiner and Lester (1972) reported evidence for the operation of the first two of these reactions in *Sacch. cerevisiae* and the same workers (Waechter *et al.*, 1969; Steiner and Lester, 1970) were able to detect enzymes that catalyse methylation of phosphatidylethanolamine, using S-adenosylmethionine as a methyl-group donor.

There are reasons for believing that *Sacch. cerevisiae* can synthesize phosphatidylethanolamine and phosphatidylcholine in a different way, using the reactions in which CDP-ethanolamine or CDP-choline reacts with a diglyceride. Indeed the fact that provision of exogenous ethanolamine or choline in the growth medium enhances synthesis of phosphatidylethanolamine and phosphatidylcholine respectively provides evidence for the operation of the cytidine nucleotide pathway (Fig. 5). Evidence for the existence of this pathway in *Sacch. cerevisiae* has been provided by Waechter *et al.* (1969), Waechter and Lester (1971) and Ratcliffe *et al.* (1973). However, the enzymatic basis of the reactions has not yet been established. There is also the suggestion by Magnall and Getz (1973) that the cytidine nucleotide pathway may be confined to mitochondria. Nevertheless, the role of the cytidine nucleotide pathway for the synthesis of phosphatidyl-ethanolamine and phosphatidylcholine will not be resolved until the enzymatic basis of the reactions has been classified.

The present thesis reports a study on the effect of enriching the plasma membrane of *Sacch. cerevisiae* with phosphatidylcholine or phospha-tidylethanolamine on the sensitivity of the cell to an anionic, a cationic and a non-ionic surfactant. It exploits the discovery, already reported, that aerobic growth in the presence of choline or ethanolamine changes the proportions of phosphatidylcholine and phosphatidylethanolamine synthesized by *Sacch. cerevisiae*. It also uses a strain of *Sacch. cerevisiae* (NCYC 366) that is particularly susceptible to the action of β-glucanases and so can be easily converted into sphaeroplasts.

Table 1. Phospholipid composition of cells and plasma membranes of *Saccharomyces cerevisiae* NCYC 366 grown in medium supplemented with choline or ethanolamine.

Phospholipid	Percentage composition with cells grown in: Choline-supplemented medium		Ethanolamine-supplemented medium	
	Cells	Membranes	Cells	Membranes
Phosphatidylcholine	46.2	32.5	35.0	25.7
Phosphatidylethanolamine	27.8	18.6	40.7	36.5
Phosphatidylinositol + phosphatidylserine	13.9	27.2	10.7	25.3
Cardiolipin	3.8	ND [a]	3.6	ND
Phosphatidic acid	3.8	18.3	4.7	6.9
Lysophospholipids	3.8	ND	4.4	ND
Base spot	0.9	3.5	0.9	5.7

[a] ND indicates that the phospholipid could not be detected.

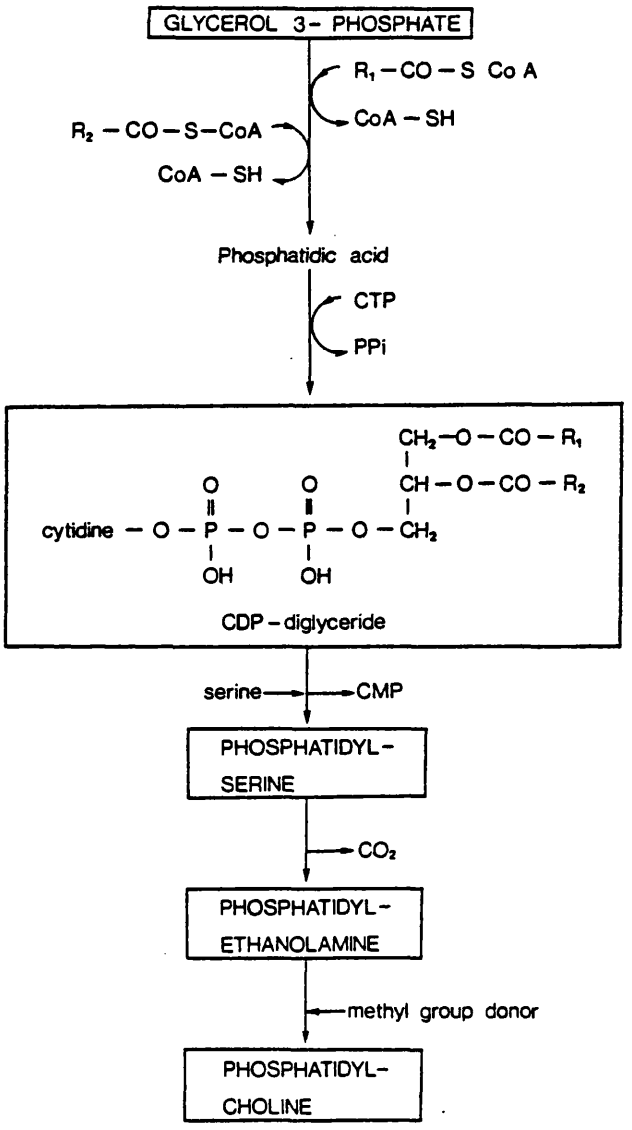


Figure 4. The Methylation Pathway.

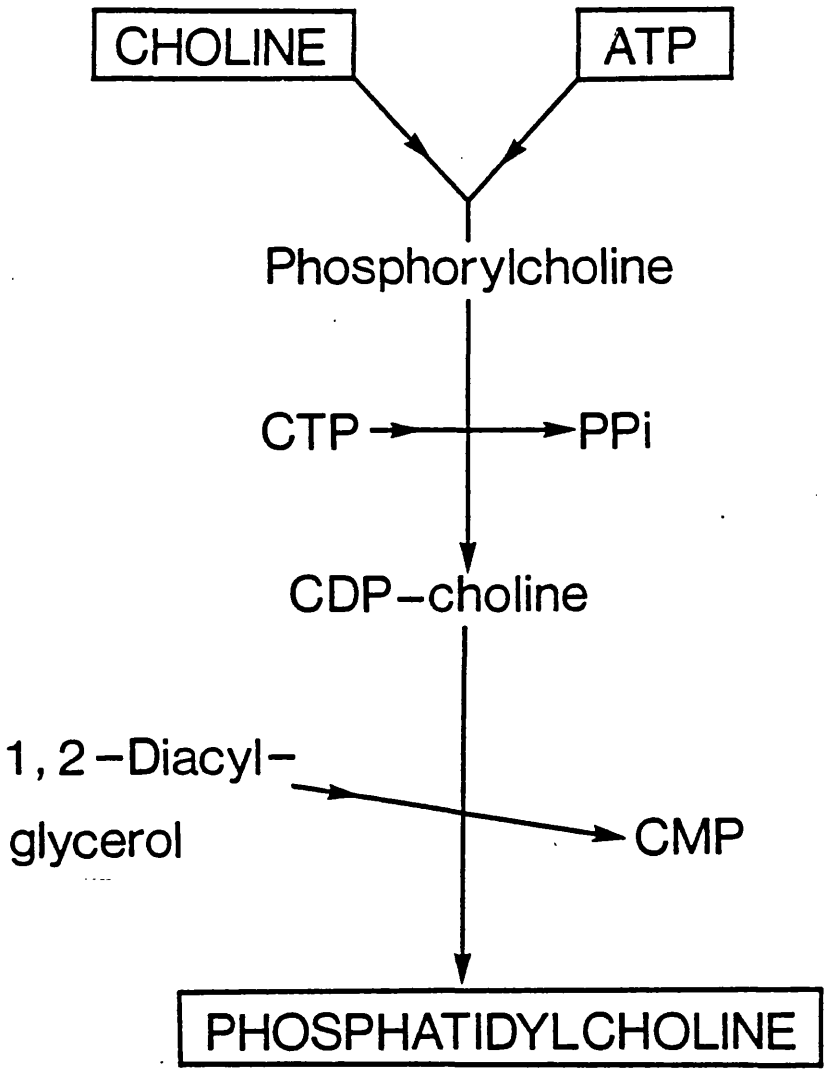


Figure 5. The Cytidine Nucleotide Pathway.

MATERIALS

AND

METHODS

MICROBIOLOGICAL METHODS

Organism

The organism used throughout this work was *Saccharomyces cerevisiae* NCYC 366, which was isolated by A.E. Wiles in 1948 from the pitching yeast of the Exchange Brewery, Sheffield, England. Slant cultures were maintained at 4°C on slopes of malt extract (3 g/l) - yeast extract (3 g/l) - glucose (10 g/l) - peptone (0.5 g/l), - agar (20 g/l) (MYGP) medium (Wickerham, 1951) which was sterilized at 121°C for 15 min.

Experimental Cultures

Cultures were grown in a glucose-salts-yeast extract medium containing per litre:-

Glucose	20.0g
$(NH_4)_2SO_4$	3.0g
KH_2PO_4	3.0g
Yeast extract (Oxoid)	1.0g
$MgSO_4.7H_2O$	25.0 mg
$CaCl_2.6H_2O$	25.0 mg

This medium was sterilized at 115°C for 10 min. Where indicated this medium was supplemented with choline chloride or ethanolamine at 1 mM. Ethanolamine was sterilized by membrane filtration (0.45 μm pore size; Millipore) and aseptically added to unsupplemented medium, while choline chloride was included in the medium prior to sterilization.

Starter cultures were prepared by inoculating 100 ml unsupplemented medium in 250 ml conical flasks from a slope culture. These shake-flasks were incubated at 30°C in a Gallenkamp orbital shaker at 250 rev./min for about 24 hours. Larger batch cultures were grown in one litre

amounts of medium in two litre round flat-bottomed flasks. They were inoculated with 0.8 mg dry wt. cells from a starter culture, and incubated overnight in a Perspex water bath at 30°C, stirred and aerated with a magnetic stirrer at 2500 rev./min as described by Patching and Rose (1969). Except where stated otherwise, cells were harvested after about 18 hours growth from late exponential-phase cultures (0.22 - 0.24 mg dry wt./ml) by centrifugation in a MSE 'High Speed 18' refrigerated centrifuge at 4°C for one min at 12250 *g*. Cells were washed according to the details in the separate experiments.

Estimation of Growth

A PYE Unicam series 2 spectrophotometer was used to estimate the dry weight of cells in cultures by measuring the absorbances (E^{600}_{1cm}) in glass cells against a water blank. Absorbance readings were related to dry weight of cells using a calibration curve ranging from 0 - 0.3 mg dry wt. Dense cultures were diluted appropriately with water.

EFFECTS OF SURFACTANTS

Effects on Growth

A range of concentrations of surface-active agents was included in 100 ml portions of unsupplemented medium. Both Triton X-100 (mol. wt. assumed to be 628; Inque and Kitagawa, 1976) and CTAB were sterilized by membrane filtration (0.45 µm pore size; Millipore), while SDS was sterilized by autoclaving at 115°C for 10 min. Media were inoculated with 0.1 mg dry wt. of cells estimated as already described. Flasks were incubated in a Gallenkamp orbital shaker (250 rev./min) at 30°C

Effects on Viability

Apart from the late-exponential phase cultures, where indicated some cultures were harvested after 24h (0.7 - 0.8 mg dry wt./ml) and after 36h (1.3 - 1.5 mg dry wt./ml). The effect of surfactants on the viability of populations was assessed by suspending cells, washed three times with 67 mM-KH_2PO_4 buffer (pH 4.5: sterilized at 115°C for 10 min), at a concentration of 0.16 mg dry wt./ml in 100 ml of the same buffer. The 250 ml flasks containing the buffer were supplemented with surfactant and shaken at 250 rev./min at 30°C in a Gallenkamp orbital shaker. Portions of this suspension were removed at times indicated, diluted appropriately with 67 mM-KH_2PO_4 buffer and portions (0.1 ml) of the diluted suspension spread on MYGP-agar plates in quadruplicate. The dilutions were adjusted throughout the experiment, as the numbers of viable cells declined, to give counts of between 30 and 300 colonies per plate. Plates were incubated at 30°C for 48h and the number of colonies on each plate counted. Viabilities are expressed as a percentage of the initial count. This procedure was carried out twice on each of two batches for cells enriched in both PC or PE to give four replicates for each supplementation.

PHOSPHOLIPID ANALYSES

Extraction of Lipids

Lipids were extracted from freeze-dried cells which had been washed three times in water, using the method of Letters (1968b) as modified by Hossack and Rose (1976). A portion of freeze-dried cells (240 mg) was suspended in hot ethanol (80% v/v; 20 ml) and maintained at 80°C for 15 min to inactivate phospholipases. The suspension was then filtered

through a Whatman no. 44 paper, washed with chloroform-methanol (2:1), and the filtrate stored at -20°C. The residue was then suspended in methanol (15 ml) for 10 min to disperse the cells before adding an equal volume of chloroform. The suspension was then stirred at room temperature (18 - 22°C) for three hours, filtered again, and the residue extracted as before for another three, followed by another two, hours. Butylated hydroxytoluene (0.005% w/v; 1 ml) was added to the combined extract to prevent oxidation of lipids. The extract was filtered through a sintered glass funnel (no. 5 porosity) and evaporated to dryness *in vacuo* using a rotary evaporatory (Büchi) and a Rotary Evapo-mix (Buchler).

Non-lipid material was removed from the extract by a modification of the procedure of Folch *et al.* (1957). The extract was dissolved in chloroform-methanol (2:1 v/v; 5 ml), 1 ml of KCl solution (0.88 w/v) added and the two phases mixed well. The extract was centrifuged at 1100 *g* for 5 min to facilitate separation. The aqueous phase was removed and discarded and the interphase washed three times with the aqueous phase of chloroform-methanol 0.88 (w/v) KCl (8:4:3 v/v/v, 1.5 ml). The purified extract was then evaporated to dryness as before and dissolved in a small volume of chloroform-methanol (2:1 v/v; 2 ml).

Separation of Phospholipids

Phospholipids were separated from other clases of lipid by quantitative thin-layer chromatography on glass plates coated with 0.4 mm of

Kieselgel $HF_{254 + 366}$ (Type 60: Merck). The plates were not activated before use. A portion of the purified extract was applied to the plates before developing with light petroleum (40 - 60°C)-diethyl ether-acetic acid (70:30:2 v/v/v). The different types of lipid were visualized using ultraviolet radiation at 254 nm and identified as described by Hossack and Rose,(1976). The phospholipid band on the plate was scraped off using an acid-washed microscope slide. Phospholipids were eluted from the silica gel by two volumes of chloroform-methanol-water (5:5:1 v/v/v; 3 ml) followed by one portion (3 ml) of methanol, and finally 3 ml of methanol-acetic acid-water (95:1:5 v/v/v). The phospholipid extract was then evaporated to dryness and dissolved in a known volume of chloroform-methanol (2:1, v/v).

Phospholipid Estimation

The phospholipid content of extracts was determined by assaying the phosphorus content of a portion (40 µl) using the method of Chen *et al.* (1956). From a standard curve the phosphorus content of the sample was calculated and, by multiplying by 25, the total phospholipid content (mg/ml) of the extract calculated. The factor of 25 converts the atomic weight of phosphorus to the molecular weight of phosphatidylcholine which is taken to be a typical phospholipid of average molecular weight.

SURFACTANT-INDUCED RELEASE OF CATIONS

Preparation of Sphaeroplasts

Sphaeroplasts were prepared by the method described by Alterthum and Rose (1973) as modified by Cartledge and Rose (1973) to include the use of imidazole (10 mM) - $MgCl_2$ (10 mM) buffer (pH 6.0) containing 1.2 M - sorbitol. Moreover Zymolyase-5000 (Kitamura *et al.*, 1971; Kitamura and Yamamoto, 1972) was used at 0.4 mg/6 mg dry wt. cells instead of a preparation of β-glucanase from Basidiomycete QM 806. Although the absorbance of the reaction mixture dropped rapidly when diluted with water, showing no further drop after 30 min, the suspension was routinely incubated for 60 min. After sphaeroplast formation was complete, they were twice washed gently in buffered 1.2 M-sorbitol(lacking $MgCl_2$), resuspended in the same buffer and used immediately or stored at $4^{\circ}C$.

Preparation of Liposomes

A portion of chloroform containing 37.5 μmoles of a mixture of yeast phospholipids or commercial phospholipids was dried in a 50 ml flask on a Büchi rotary evaporator at 45 - $50^{\circ}C$. The last traces of solvent were removed from the flask by flushing with nitrogen gas for 1 min. A portion (3 ml) of imidazole-HCl (50 mM; pH 6.0) buffer containing 150 mM-KCl was added to the flask,the head space flushed with nitrogen gas and the flask gently turned by hand until all of the phospholipid was removed from the walls of the flask. Resuspension of the phospholipid was aided by including two glass beads (2.0 mm diam.) in the flask. The suspension of liposomes was subjected to ultrasound

for 5 min using a MSE. 100 watt ultrasonicator, fitted with a 3 mm diameter probe, at maximum amplitude. The tube containing the liposome suspension was immersed in an ice-water mixture whilst in the ultrasonicator, and the head space above the suspension continually flushed with nitrogen gas. The ultrasonicated suspension, which contained liposomes of a fairly uniform size (1.0 - 1.5 μm diam.), was then dialysed in visking tubing for 2 - 3 h against imidazole-HCl buffer containing 150 mM-$MgSO_4$, the buffer being changed three times during this period. Suspensions of liposomes were used immediately or stored at 4°C.

Measurement of Ion Release

Release of ions from cells, sphaeroplasts and liposomes was measured using an EIL 7050 pH/selective ion meter (Electronic Instruments Ltd., Chetsey, Surrey) fitted with an EIL K^+-selective glass electrode (33 1057 200) and a sulphate reference electrode (33 1057 230) coupled to a potentiometric recorder (Servoscribe RE 511.20, Smiths Industries Ltd., Wembley, Middlesex) as shown in Fig. 6. The electrode was equally sensitive to K^+ and NH_4^+, and 25% as sensitive to Na^+. Measurements were carried out in plastic tubes placed in a jacketed water bath, maintained at 25°C, and the contents agitated by a magnetic stirrer. Cells and sphaeroplasts were washed twice in imidazole-HCl buffer (10 mM; pH 6.0) containing 1.2M-sorbitol and a portion containing 5 mg dry wt. equiv. suspended in the same buffer. After the recorder base line had stabilized, the suspension was supplemented with buffer containing surfactant so that the final volume of the suspension was 10 ml. Liposomes were suspended

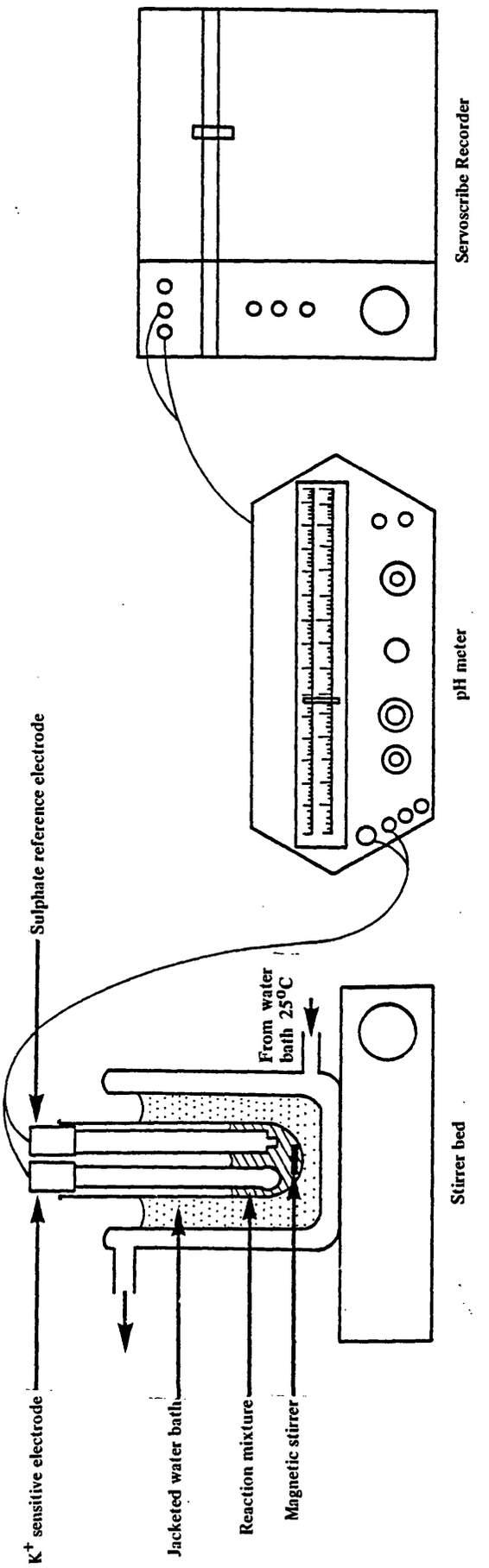


FIG. 6 Apparatus for detecting release of cations

in imidazole-HCl (50 mM; pH 6.0) containing 150 mM-$MgSO_4$ at 1.0 µmole phospholipid equiv. giving a final volume of 10 ml. after addition of the surfactant. The instrument was calibrated using standard solutions of KCl, and the release of ions from cells, sphaeroplasts and liposomes expressed as percentage released of the total content of K^+ or K^+ equivalents. Since the Na^+ from SDS interfered with measurement of the release of K^+ equivalents, its contribution was determined using a buffer blank, and the responses to SDS of the test samples corrected using the response of the blank. The content of K^+ equivalents in cells was determined by placing a standard suspension (5 mg dry wt. in 10 ml) in a bath of boiling water for 30 min, cooling, and measuring the content of K^+ equivalents in the suspension after making up to 10 ml. The total cation content of liposomes and sphaeroplasts was determined by incorporating Triton X-100 at 1.0 mM. All solutions were made up using deionized glass-distilled water.

EXPERIMENTS ON CELL WALLS

Preparation of Walls

Walls from freshly harvested cells were prepared by a modification of the method described by McMurrough and Rose (1967). Cells (about 240 mg dry wt.) from a one litre culture were centrifuged and washed three times in 67 mM-KH_2PO_4 at 4°C. They were then suspended in about 15 ml buffer, and added to 40 g of Ballotini beads (0.17 - 0.18 mm diam.). The suspension was then treated in the Braun cell homogenizer (B. Braun, Melsungen, West Germany) for 4 min, while

cooling the glass homogenizer bottle with carbon dioxide. The suspension of disrupted cells was filtered through a sintered glass funnel (no. 1) and the beads which were retained on the sinter washed several times with buffer. Washings were pooled with the filtrate, the suspension centrifuged and the supernatant liquid rejected. The pellet was then washed with 0.9% (w/v) NaCl three times or until the supernatant liquid became clear. The pellet was then washed with buffer into a tapered centrifuge tube. After centrifugation, the pellet was made up of two distinct layers. The upper white layer formed about two thirds of the total volume of the pellet and contained walls. This material was separated from the cream-coloured bottom layer by adding ice-cold water and gently shaking the tube until the material in the upper layer was almost completely suspended. Up to this stage, all operations were conducted under ice-cold conditions. The suspension of walls in water was then placed in a bath of boiling water for 10 min. After cooling, the walls were washed at least 10 times in water, and freeze dried (Edwards Freeze Drier; Model 30P2/727).

Analysis of Walls

Determination of Carbohydrate. Glucan and mannan contents of walls were determined by the method of McMurrough and Rose (1967), which is a modification of the sulphuric acid-carbazole differential extinction method originally described by Dische (1927, 1930), and later embellished by Gurin and Hood (1939), Siebert and Atno (1946), and Holzman *et al.* (1947).

A portion of wall (3 mg) was dissolved in 1 ml of sulphuric acid (70% v/v) which was then made up to 10 ml with water. Duplicate portions (0.5 ml) of this solution were dispensed into acid-washed boiling tubes and aqueous sulphuric acid (5.0 ml; 8 vol. conc. H_2SO_4: 1 vol. H_2O) was added carefully to each tube. The tubes were vigorously shaken and allowed to cool to room temperature. Carbazole reagent (0.3 ml, containing 0.5% (w/v) carbazole in 95% (v/v) ethanol was added to each tube. The tubes were covered with aluminium foil, the contents rapidly mixed and the tubes placed in a boiling water bath for 10 min. After cooling, the extinctions of the solutions were measured at 435 (R_1) and 535 (R_2) nm in glass cuvettes (1 cm) in a PYE Unicam SP600 series 2 spectrophotometer. The following equations were used to estimate the amounts of glucose and mannose.

$$\text{Mannose (mg)} = \frac{R_1Y - R_2A}{XY - AB}$$

$$\text{Glucose (mg)} = \frac{R_2X - R_1B}{XY - AB}$$

where X = extinction for 1 mg mannose at 435 nm; Y = extinction for 1 mg glucose at 535 nm; A = extinction for 1 mg glucose at 435 nm; and B = extinction for 1 mg mannose at 535nm.

Protein Estimation. Protein was extracted from walls (3 mg dry wt.) in an acid-washed tube by adding 1.5 M-NaOH (0.2 ml) and 0.4 ml H_2O, sealing the tube, and placing the tubes in a steam bath for 15 min.

Protein in the extract was determined by the method of Lowry *et al.* (1951). Standard curves were prepared relating extinction at 750 nm to protein content (within the range 25 - 500 µg) using bovine serum albumin (Sigma).

Porosity of Walls and Membranes

The porosity of walls and membranes of cells enriched with either PC or PE was determined using a range of polyethylene glycols of finite number - average molecular weight as described by Scherrer *et al.* (1974). Cells were harvested, washed twice with water and then transferred to a pre-weighed Corex tube and centrifuged at 20000 *g* in a MSE 'High Speed 18' centrifuge at 4°C for 45 min. The supernatant was decanted and all excess water removed from the inside of the tube with tissue paper. After weighing, 2 ml of solute (3% w/v solution) was added to the tube containing the pelleted cells (about 2 g) and this vortex mixed. The mixture was then left to equilibrate for 2 hours at 4°C, after which time it was centrifuged as before. A small portion of supernatant was removed and the concentration of the solute measured using a 'High Accuracy' Abbe 60 refractometer (Bellingham and Stanley, London). From these measurements values for the total uptake (S^W) and the uptake corrected for interstitial space (R^W) were calculated. The maximum intracellular space was taken to be identical with the percentage uptake of tritiated water, while the interstitial space was determined using Dextran 5000 (Sigma) with a number-average molecular weight of 500,000. Uptake of tritiated water was determined by adding 0.1 ml.of supernatant, made up to 0.5 ml with water, to 20 ml.

of scintillation fluid (Unisolve 1). The vials were then left at least 4 hours before counting in a liquid scintillation counter (model 6500; Nuclear Enterprises Ltd. Reading, Berks.).

Measurement of Electrophoretic Mobility

The electrophoretic mobility of cells at different pH values was measured using a Carl Zeiss Cytopherometer (Carl Zeiss, Oberkochen, West Germany) using a modification of the method of Somers and Fisher (1967). Movement of the cells was timed over 80 μm in both directions (current reversed). Each mobility value was the mean of at least 20 observations. Mobility was measured at 30°C using yeast suspensions, in the appropriate buffer, containing 0.5 mg dry wt. equiv. or 10^7 organisms per ml. The buffers used in these experiments (Gittens and James, 1963) were: pH 2.0 - NaCl/HCl pH 3.0 - 9.0 NaCl/ Sodium acetate/Sodium barbiturate/HCl. However these buffers were not prepared as described by Gittens and James (1963), but by diluting a stock solution to an ionic strength of 0.05 and adjusting to the required pH value using HCl with an ionic strength of 0.05. The conductivity of buffers was measured with a MEL conductivity bridge (The MEL Equipment Co. Ltd., England).

ELECTRON MICROSCOPY

Scanning Electron Microscopy

Cells were washed twice in 50 mM-phosphate buffer (pH 7.0) and fixed in the same buffer containing 3% (w/v) glutaraldehyde for one hour with occasional agitation. After centrifugation, the cells

were dehydrated for 10 min in 50% v/v) then 70% (v/v) ethanol and finally three times in absolute ethanol for 15 min. The fixed and dehydrated cells were then resuspended in acetone and dried in a Polaron E 5000 critical point drier (Polaron Equipment Ltd., Watford, Herts). A small portion of dried cells was attached to a conductive stub with double-sided cellulose tape and sputter coated with gold in the presence of argon. The specimen was examined using a Cambridge S4 Stereoscan electron microscope (Cambridge Instruments, Rustat Road, Cambridge) at an accelerating voltage of 20 KV. Photographs were taken on Ilford FP4 film which was developed in Ilford ID 11 for 10 min at 20°C, washed in water for 30 sec and fixed with Ilford Hypam for 5 min.

Sphaeroplasts and cells that had been incubated in Zymolyase-5000-containing buffer were removed from suspension by rapid centrifugation at intervals during the process. The structures were gently washed twice in buffered imidazole-HCl (10 mM; pH 6.5) containing 1.2 M-sorbitol and suspended in the same buffer supplemented with 3% (w/v) glutaraldehyde for one hour with occasional agitation. Fixed structures were washed twice in imidazole-HCl buffer containing 1.2 M-sorbitol and post-fixed in the same buffer supplemented with 2% (w/v) osmium tetroxide for a further hour with occasional agitation. After centrifugation, the structures were dehydrated and sputter coated with gold as described for cells. They were examined with a JEOL 100 CX electron microscope having a tungsten filament and fitted with JEOL ASID 'high resolution scanning device' (JEOL (UK) LTd., JEOL House, Grove Park, London). An accelerating voltage of 20 KV was used.

Photographs were taken on Ilford HP4 film which was developed in Ilford ID 11 for 7 min at 20°C, washed with water for 30 sec. and fixed with Ilford Hypam for 15 min.

Transmission Electron Microscopy

Cells, that had or had not been treated with 0.1 mM-SDS for 30 min. were washed twice in 67 mM-KH_2PO_4 buffer (pH 4.5) and fixed in the same buffer supplemented with 3% (w/v) glutaraldehyde for one hour. They were then washed once in 67 mM-KH_2PO_4 buffer and post-fixed in the same buffer supplemented with 2% (w/v) osmium tetroxide for a further hour. Suspensions were agitated occasionally during the fixation process. The fixed cells were dehydrated in a graded series of ethanol solutions as previously described. The fixed and dehydrated cells were suspended in molten (45°C) 2% agar which acted as a support during the embedding process. After the suspension had been extruded through a Pasteur pipette, the solidified agar was cut into 3 to 5 mm lengths which were dehydrated by several changes in absolute ethanol. The dehydrated pellets were set in Spurr's low-viscosity resin (Spurr, 1969). The pellets were tumbled overnight in resin using a Polaron tumbler 1010, then set in plastic moulds (4 ml volume) with fresh resin and hardened at 70°C for 3 days. Sections were cut using glass knives on a Reichert OM 3 ultramicrotome (American Optical Co. Ltd., Slough). They were floated on water, stretched using chloroform vapour to give silver sections (60 to 90 nm thick) and supported by copper grids (3 mm; 80 line per cm). Staining of the sections was carried out by suspending for 5 min in uranyl acetate (a saturated

solution in 70% ethanol), after which they were rinsed in water and stained for a further 5 min in Reynolds lead citrate (Reynolds, 1963). Finally they were rinsed in water. The sections were examined using a JEOL 100 CX electron microscope with an accelerating voltage of 80 KV. Photographs were taken on Kodak 4489 electron microscope film which was developed in D19 for 4 min, at 20°C, washed with water and fixed for 5 min in Ilford Hypam.

Sphaeroplasts. After preparation and washing, sphaeroplasts were fixed as previously described for cells except that imidazole-HCl buffer (10 mM; pH 6.5) containing 1.2 M-sorbitol was used instead of 67 mM-KH_2PO_4 buffer. Apart from this change of buffer, which was needed to stabilize the osmotically sensitive structures during fixation, the procedure was exactly the same as described for cells.

MATERIALS

Chemicals and materials used during this project were supplied by the following manufacturers or agents:

Anderman and Company Limited, Battlebridge House, Tooley Street, London, (Agents for E. Merck, Darmstadt, West Germany) supplied the Kieselgel $HF_{254 + 366}$.

British Drug Houses, Poole, England, supplied cetyltrimethylammonium bromide, choline chloride, ethanolamine, sodium dodecyl sulphate (specially pure), Triton X-100 and glutaraldehyde.

V.A. Howe and Company Limited, 88 Peterborough Road, London SW6, (Agents for Millipore Filter Corporation, Bedford, Massachusetts, U.S.A. and Sartorius-membranfilter GmbH, 34 Göttingen Postf. 142, West Germany) for membranes used in sterilization (0.45 µm pore size) and membrane filters used in the cytopherometer (Type SM 11539).

Kirin Brewery Company Limited, Takasaki, Gumma Pref. Japan, supplied the Zymolyase-5000.

Koch-Light, Colnbrook, Buckinghamshire, England, supplied glycols, and polyethylene glycols and liquid scintillator, Unisolve 1.

The Radiochemical Centre, Amersham, Buckinghamshire, England, supplied the tritiated water.

Sigma Chemical Company Limited, Kingston upon Thames, Surrey, England, supplied bovine serum albumin, phosphatidic acid, phosphatidylcholine and phosphatidylethanolamine.

Taab Laboratories, 52 Kidmore End Road, Emmer Green, Reading, England, supplied copper electron microscope grids, osmium tetroxide and the components of Spurr's resin.

All other chemicals were analytical grade (i.e. AnalaR or AR) or of the highest purity commercially available. Chloroform, ethanol and methanol were redistilled before use.

Laboratory glassware was routinely cleaned by soaking for at least 24h in 'Decon 75' (2%, v/v), rinsed twice in tap water and finally twice in glass-distilled water.

RESULTS

SACCHAROMYCES CEREVISIAE NCYC 366 AS A MODEL ORGANISM TO STUDY SURFACTANT SUSCEPTIBILITY

Effect of Surface-Active Agents on Growth of *Saccharomyces cerevisiae* NCYC 366

One of the first objectives was to obtain a measure of the potency of each of the three surfactants being studied. This was done by growing the organism, without supplementation, in the presence of different concentrations of each of the surfactants. Cetyltrimethylammonium bromide was found to be the most potent, inhibiting growth completely at a concentration of 5.0 μM (Fig. 7),and this was followed by SDS (Fig. 8) which severely restricted growth at a concentration of 0.10 mM. The least effective of the three surfactants was the non-ionic Triton X-100 (Fig. 9), which even at a concentration of 0.10 M did not inhibit growth to any great extent.

Effects of Surface-Active Agents on the Viability of *Saccharomyces cerevisiae* NCYC 366

Only CTAB and SDS were used to investigate the effect of surfactants on cells enriched with PC or PE since Triton X-100 did not prove to be inhibitory to growth of cells. These experiments showed a clear difference in the action of the other two surfactants on cells enriched in PC or PE. The enrichment caused little if any difference on the inhibitory effect of 0.04 mM-CTAB (Fig. 10), nor was there any detectable difference at any other concentrations of this agent. Nevertheless, the enriched cells were differently sensitive to 0.10 mM-SDS (Fig. 11), the PE-enriched cells being more sensitive than those

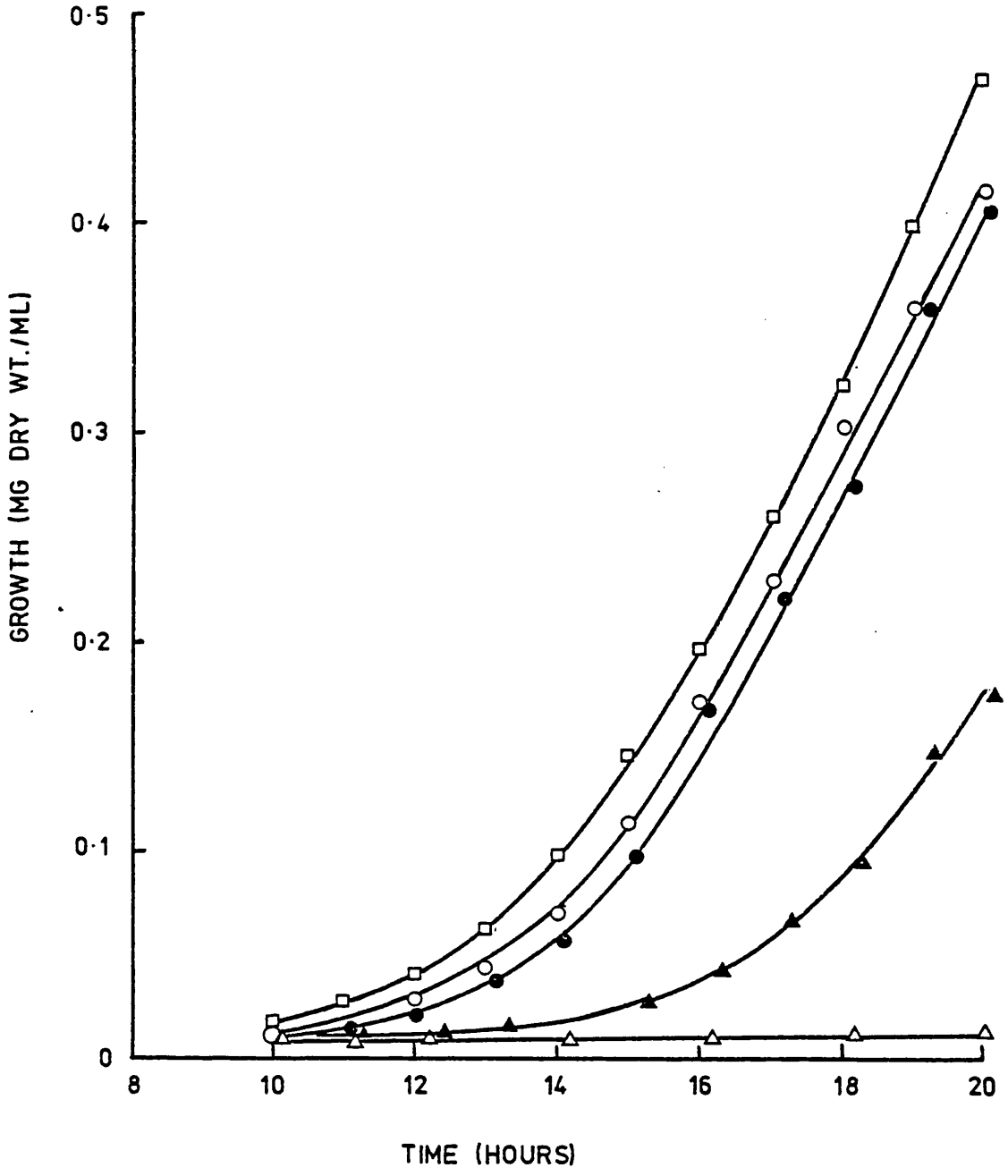


Figure 7. Time-course of growth of *Saccharomyces cerevisiae* NCYC 366 in 100ml portions of medium not supplemented with choline or ethanolamine and either lacking CTAB (□), or supplemented with CTAB at 1.0 μM (○), 1.5 μM (●), 2.5 μM (▲) or 5.0 μM (△). Values plotted are the average of four determinations.

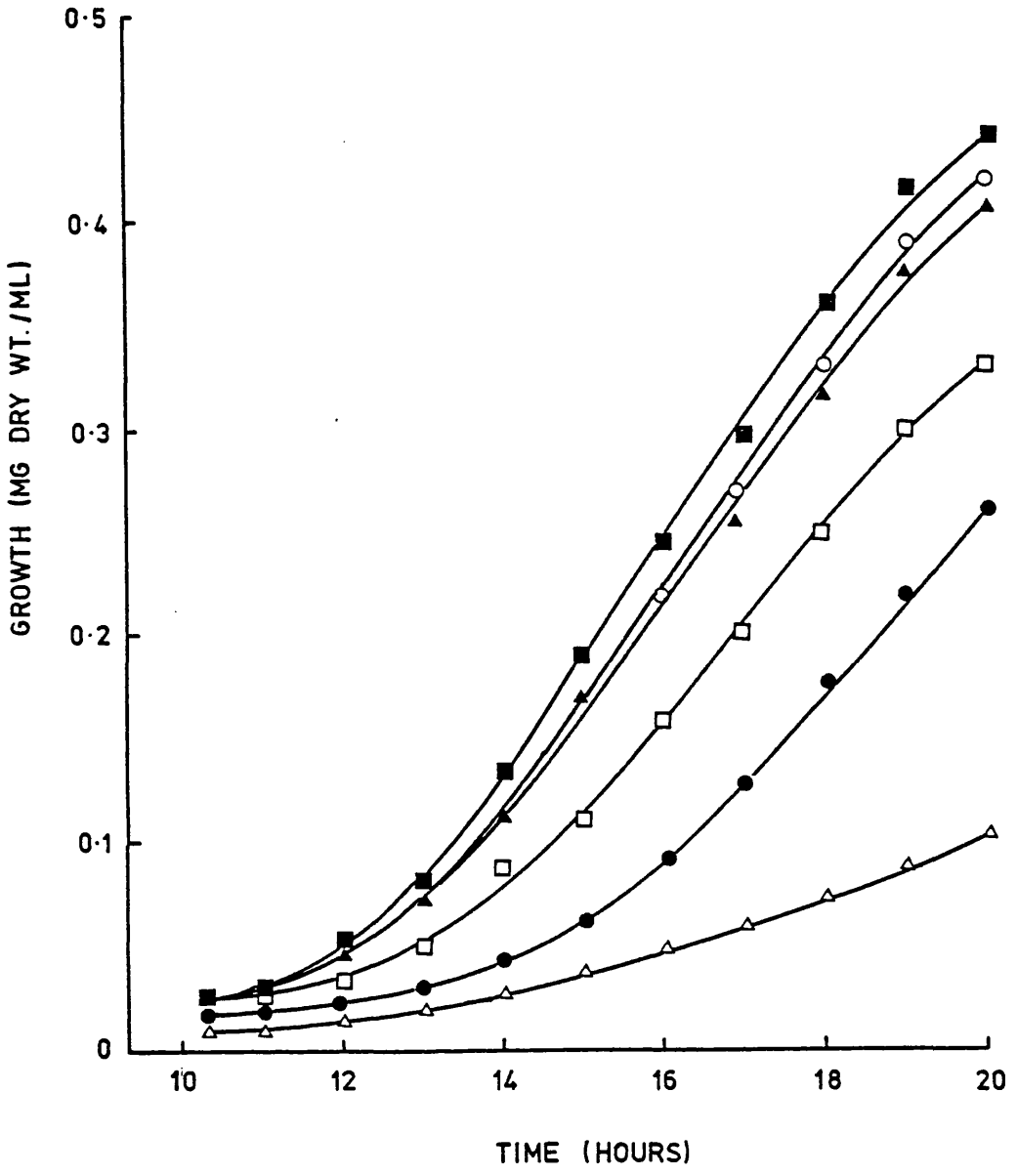


Figure 8. Time-course of growth of *Saccharomyces cerevisiae* NCYC 366 in 100 ml portions of medium not supplemented with choline or ethanolamine and either lacking SDS (■), or supplemented with SDS at 0.01 mM (○), 0.025 mM (▲), 0.05 mM (□), 0.075 mM (●) or 0.10 mM (△). Values plotted are the average of four determinations.

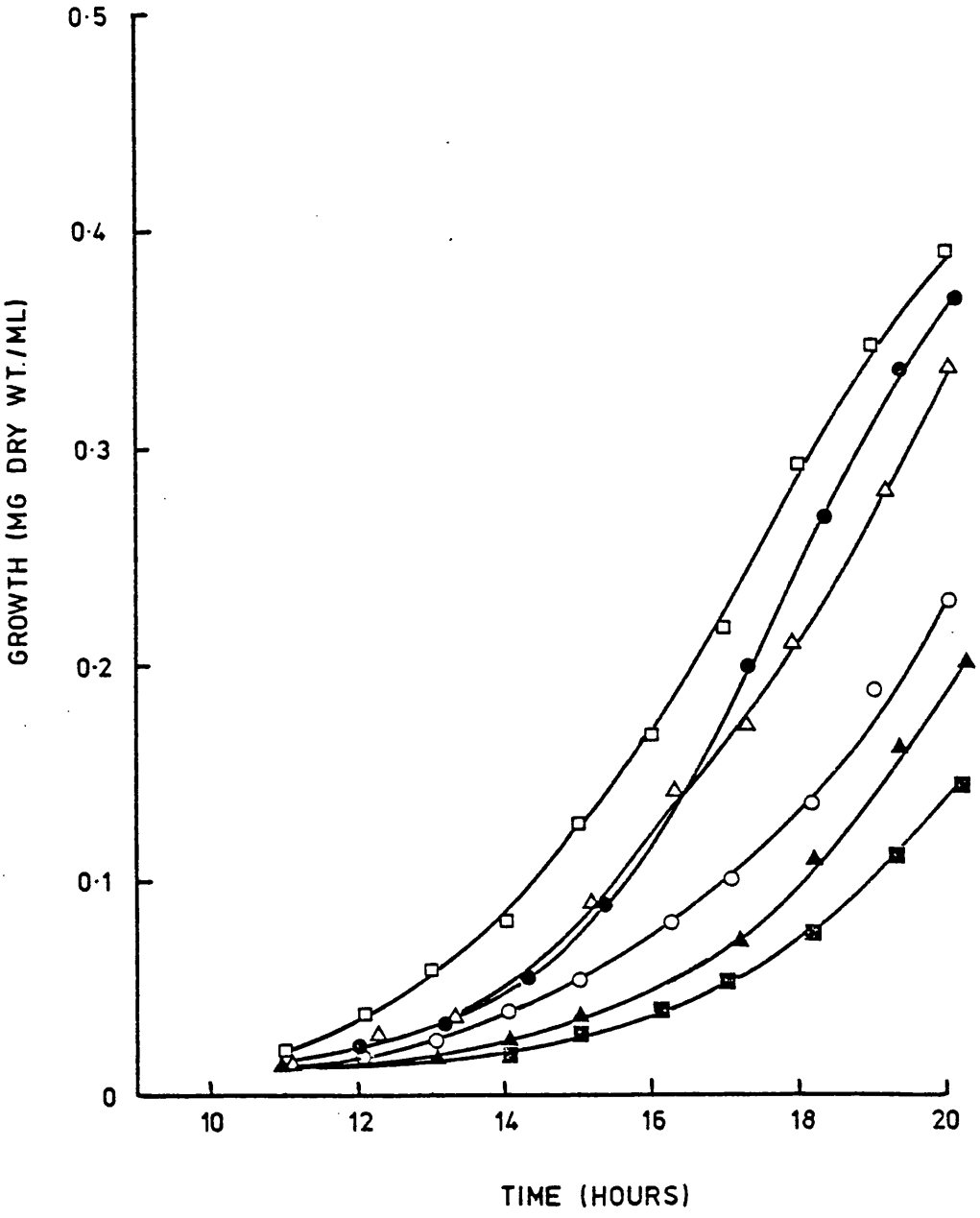


Figure 9. Time-course of growth of *Saccharomyces cerevisiae* NCYC 366 in 100 ml portions of medium not supplemented with choline or ethanolamine and either lacking Triton X-100 (□), or supplemented with Triton X-100 at 0.01 mM (●), 0.10 mM (△), 1.0 mM (○), 0.01 M (▲) or 0.10 M (■). Values plotted are the average of four determinations

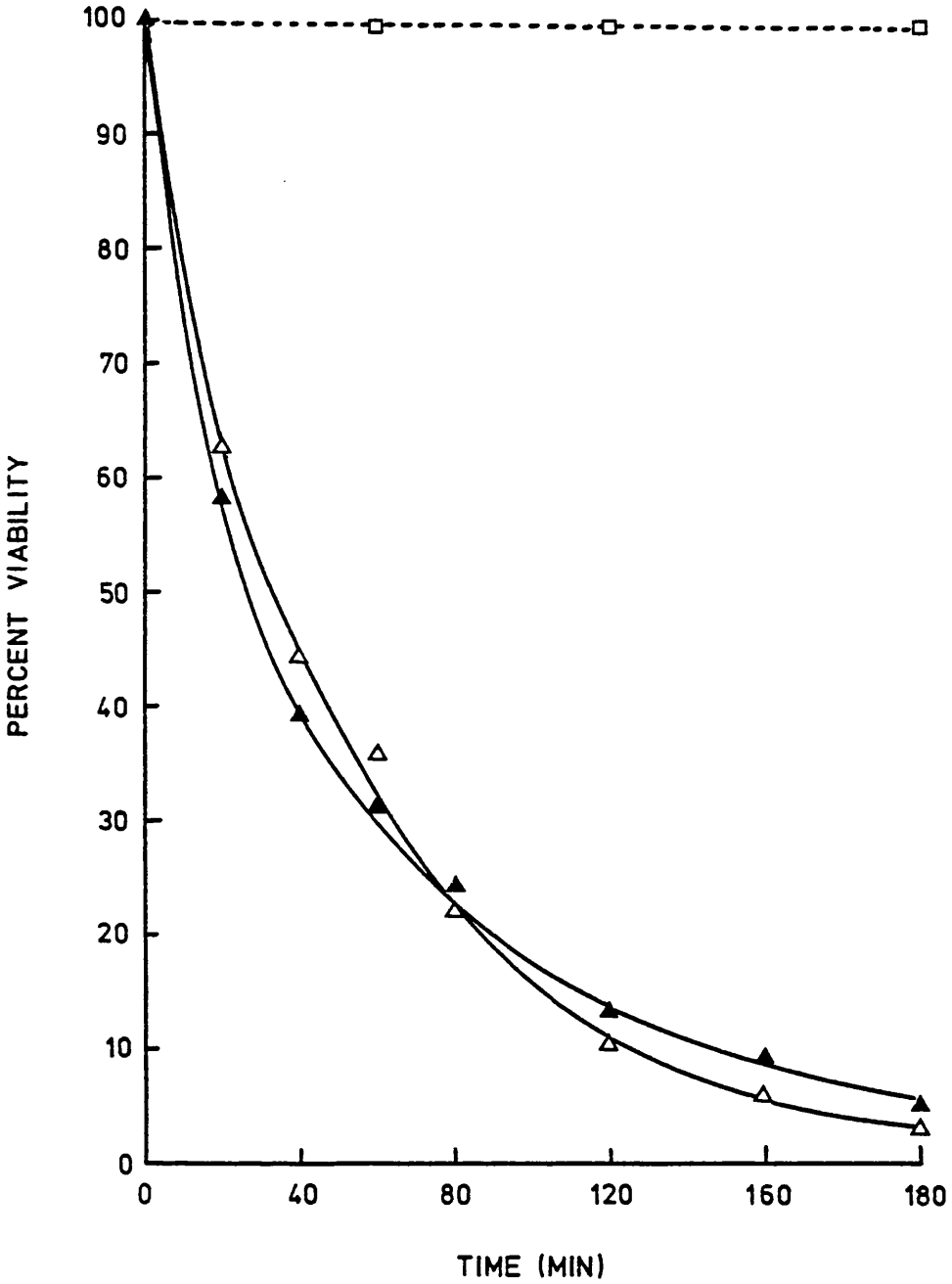


Figure 10. Decline in viability of populations of *Saccharomyces cerevisiae* NCYC 366 grown in the presence of choline (▲) or ethanolamine (△), and suspended in phosphate buffer (pH 4.5) either lacking (□, broken line) or supplemented with 0.04 mM-CTAB (continuous lines). The 95% confidence limit on the curve describing viability in PC-enriched populations is ± 3.10%, and on the curve describing viability in PE-enriched populations ± 6.93%. Values plotted are the means of four results from each of two independent determinations.

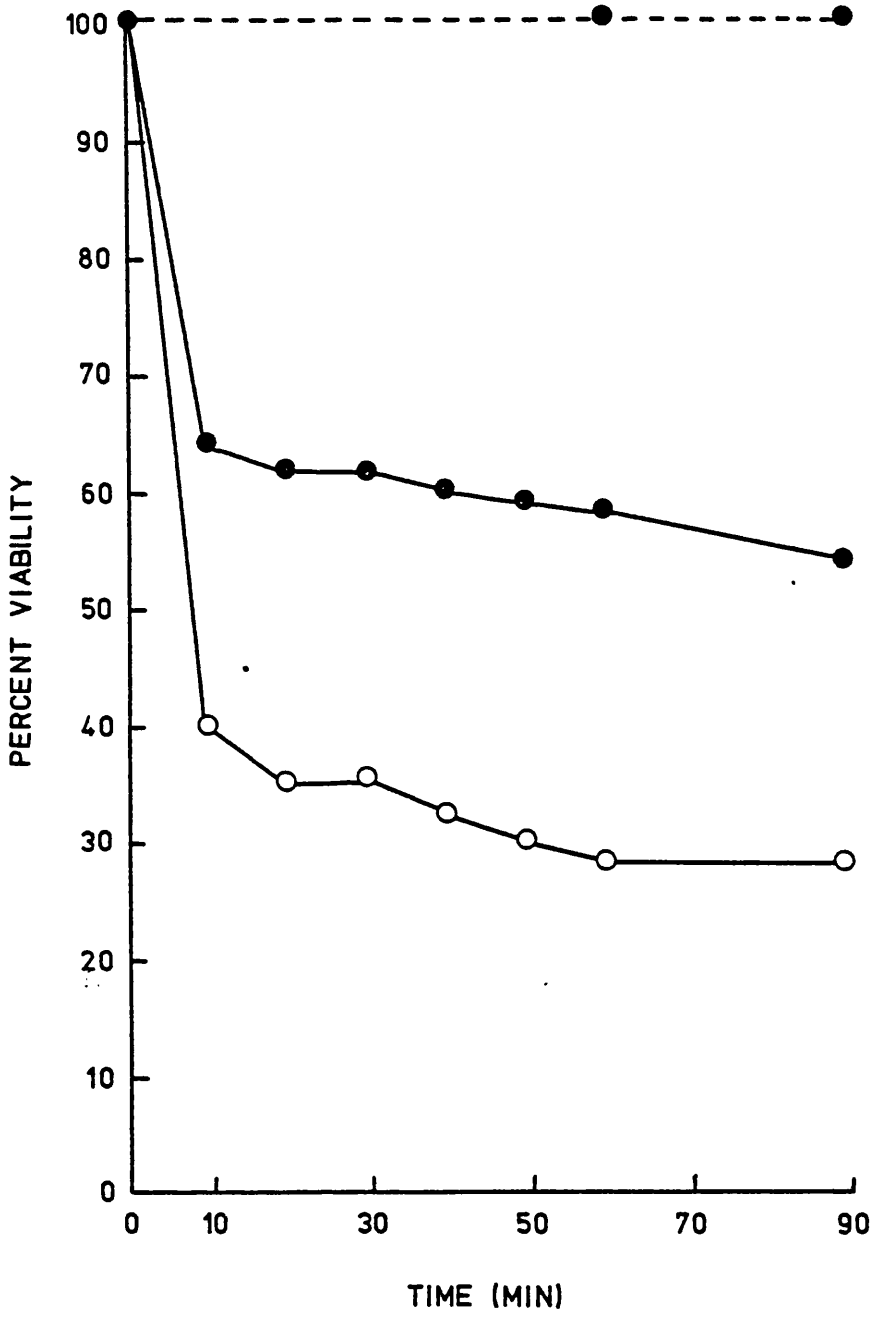


Figure 11. Decline in viability of populations of *Saccharomyces cerevisae* NCYC 366 harvested from a culture containing 0.22 - 0.24 mg dry wt./ml. Cells were grown in the presence of choline (●) or ethanolamine (○), and suspended in phosphate buffer (pH 4.5) either lacking (●, broken line) or supplemented with 0.10 mM-SDS (continuous lines). The 95% confidence limit on the curve describing viability in PC-enriched populations is ±7.67%, and on the curve describing viability in PE-enriched populations ±5.36%. Values plotted are the means of four results from each of two indpendent determinations.

enriched with PC. This differential effect of SDS on enriched cells became less marked as the age of the culture increased, as did the overall sensitivity of cells to this agent (Fig. 12). Cells enriched in PE and harvested from cultures containing 0.7 to 0.8 mg dry wt./ml (about 24 h growth) continued to show greater sensitivity to SDS compared with those enriched in PC, but cells harvested between 1.3 mg and 1.5 mg dry wt./ml (about 36 h growth) appeared insensitive to 0.1 mM-SDS regardless of the nature of enrichment.

Release of Cations Induced by Surface-Active Agents

Cells. Release of K^+ equivalents from cells enriched in PC or PE when suspended in buffered sorbitol (1.2 M) was similar whether they were challenged with 0.01 mM-CTAB or 0.25 mM-Triton X-100 (Figs. 13 and 14). The slight increase in release of cations from PC-enriched cells after 3 min induced by Triton X-100 was not significant. On the other hand, when suspensions of enriched cells were supplemented with 1.0 mM-SDS (Fig. 15), although the initial rates of release were similar, after 2.5 min there was a progressively increasing rate of cation release from PE-enriched cells. It was found necessary to incorporate 1.2 M-sorbitol into the buffer for these experiments, as washing with unsupplemented buffer caused loss of all unbound detectable cations. The least effective surfactant in causing release of cations from cells was SDS, followed by Triton X-100, and CTAB was the most potent at inducing release of cations. There was no detectable release of cations from cells suspended in surfactant-free buffer containing 1.2 M-sorbitol.

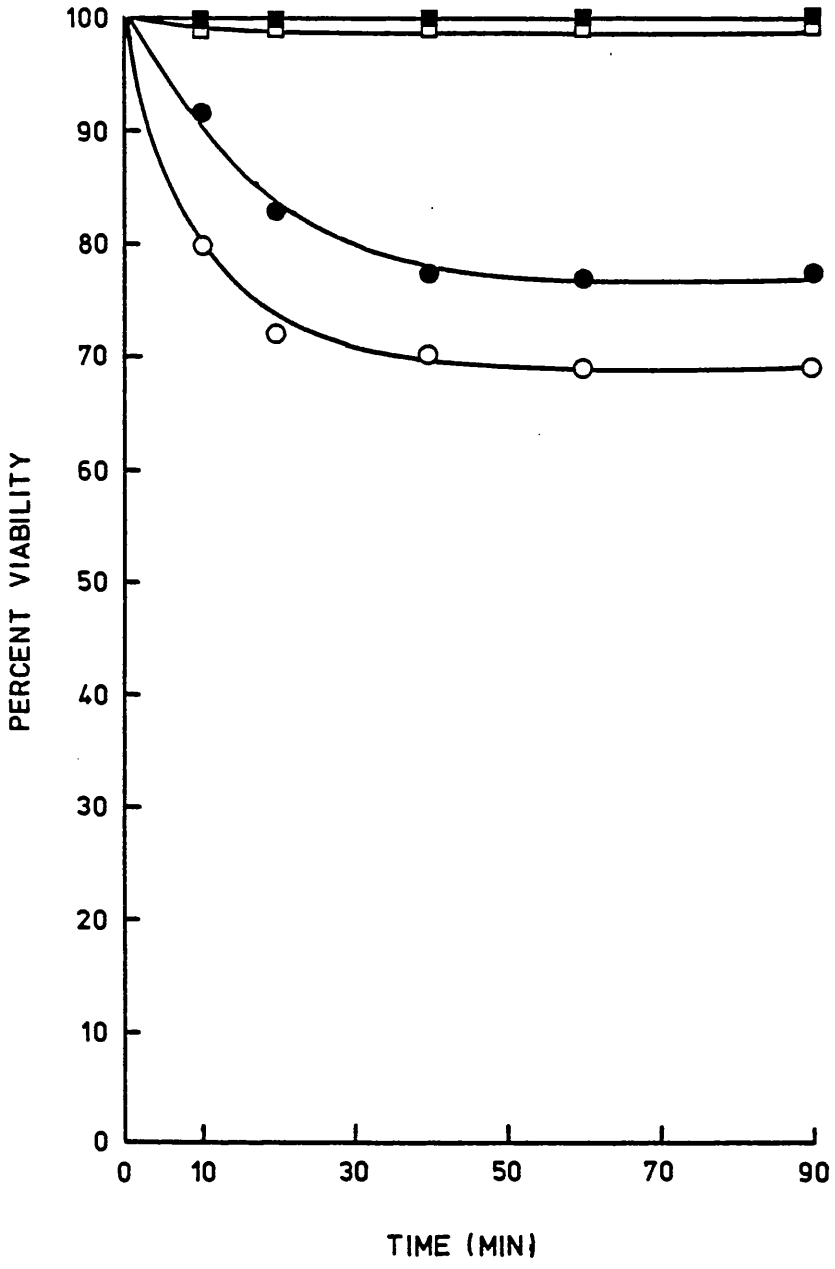


Figure 12. Decline in viability of populations of *Saccharomyces cerevisiae* NCYC 366 grown in the presence of choline (closed symbols) or ethanolamine (open symbols) and suspended in phosphate buffer (pH 4.5) supplemented with 0.10 mM-SDS. Cells were harvested at 0.7 - 0.8 mg dry wt./ml (circles) or 1.3 - 1.5 mg dry wt./ml (squares). The 95% confidence limits on the curves are ± 4.28% (●) and ±2.82% (○). Values plotted are the means of four results from each of two independent determinations.

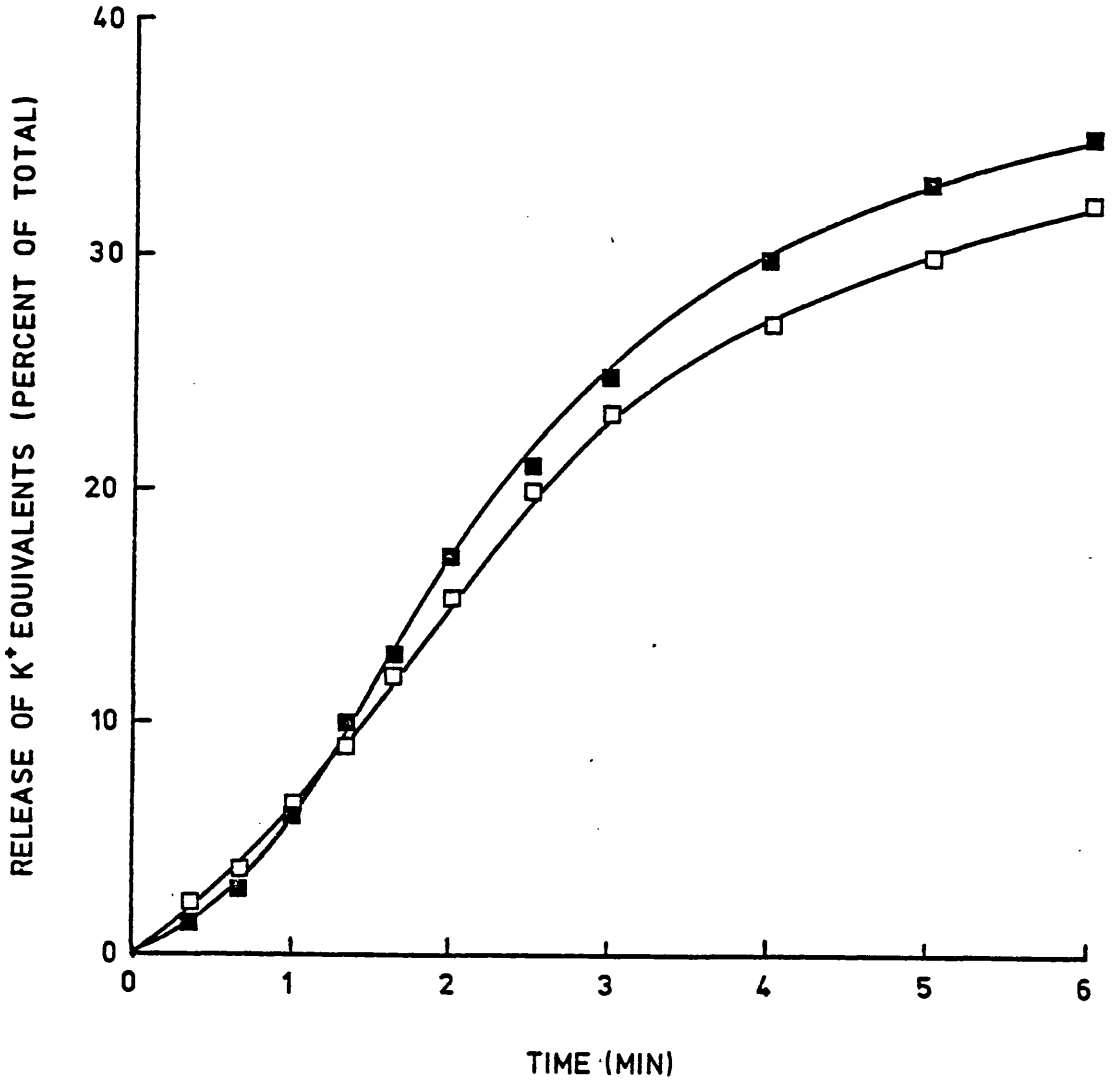


Figure 13. Time-course of release of K^+ equivalents from *Saccharomyces cerevisiae* NCYC 366 with plasma membranes enriched in PC (■) or PE (□) suspended in buffered 1.2 M - sorbitol (pH 6.0) containing 0.01 mM-CTAB. The 95% confidence limit on the curve describing the release from PC-enriched cells is ± 5.28% and from PE-enriched cells ± 4.33%. Values plotted are the average of three determinations.

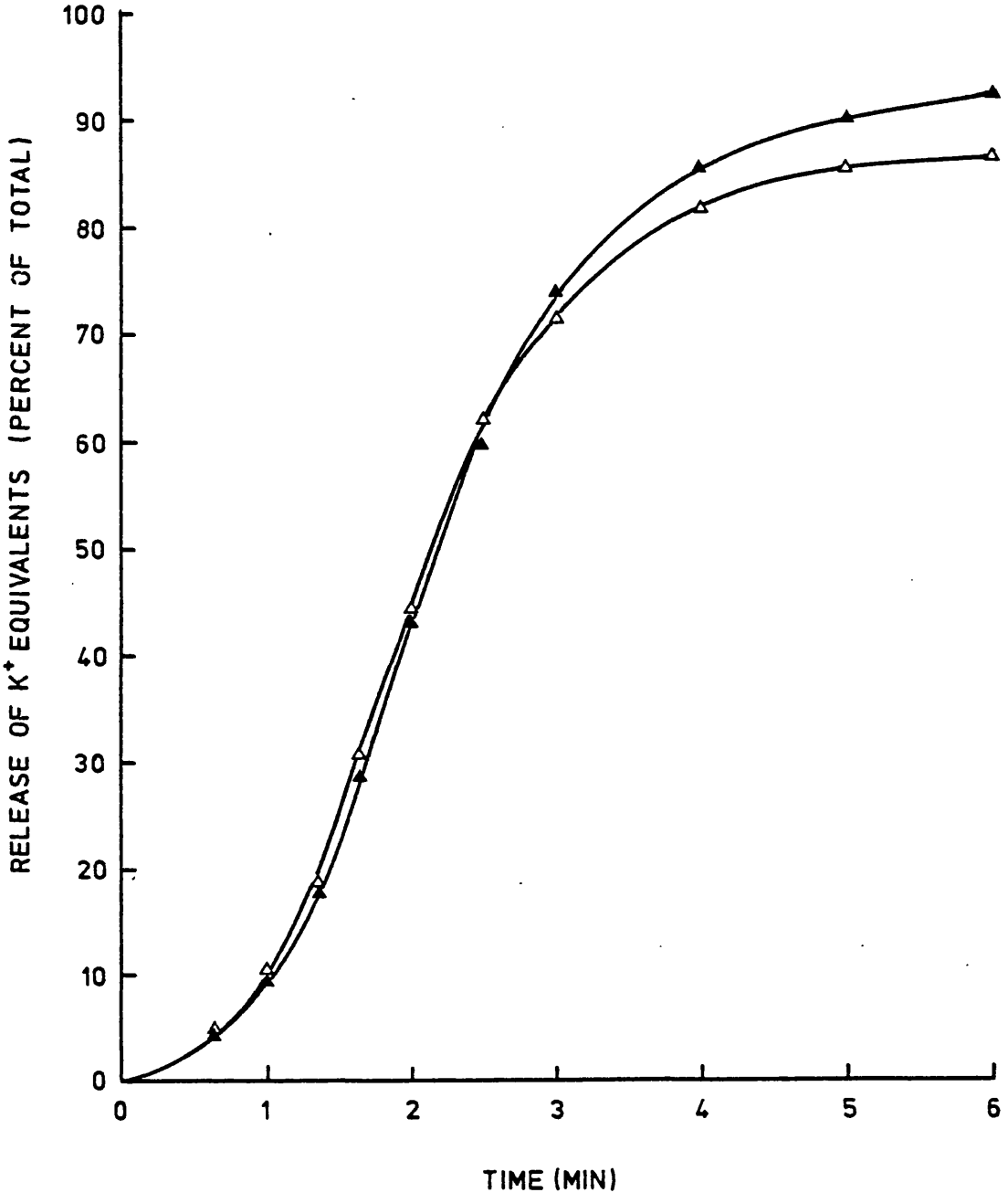


Figure 14. Time-course of release of K^+ equivalents from *Saccharomyces cerevisiae* NCYC 366 with plasma membranes enriched in PC (▲) or PE (△) suspended in buffered 1.2 M-sorbitol (pH 6.0) containing 0.25 mM-Triton X-100. The 95% confidence limit on the curve describing release from PC-enriched cells is ± 1.95% and from PE-enriched cells ± 3.56%. Values plotted are the average of three determinations.

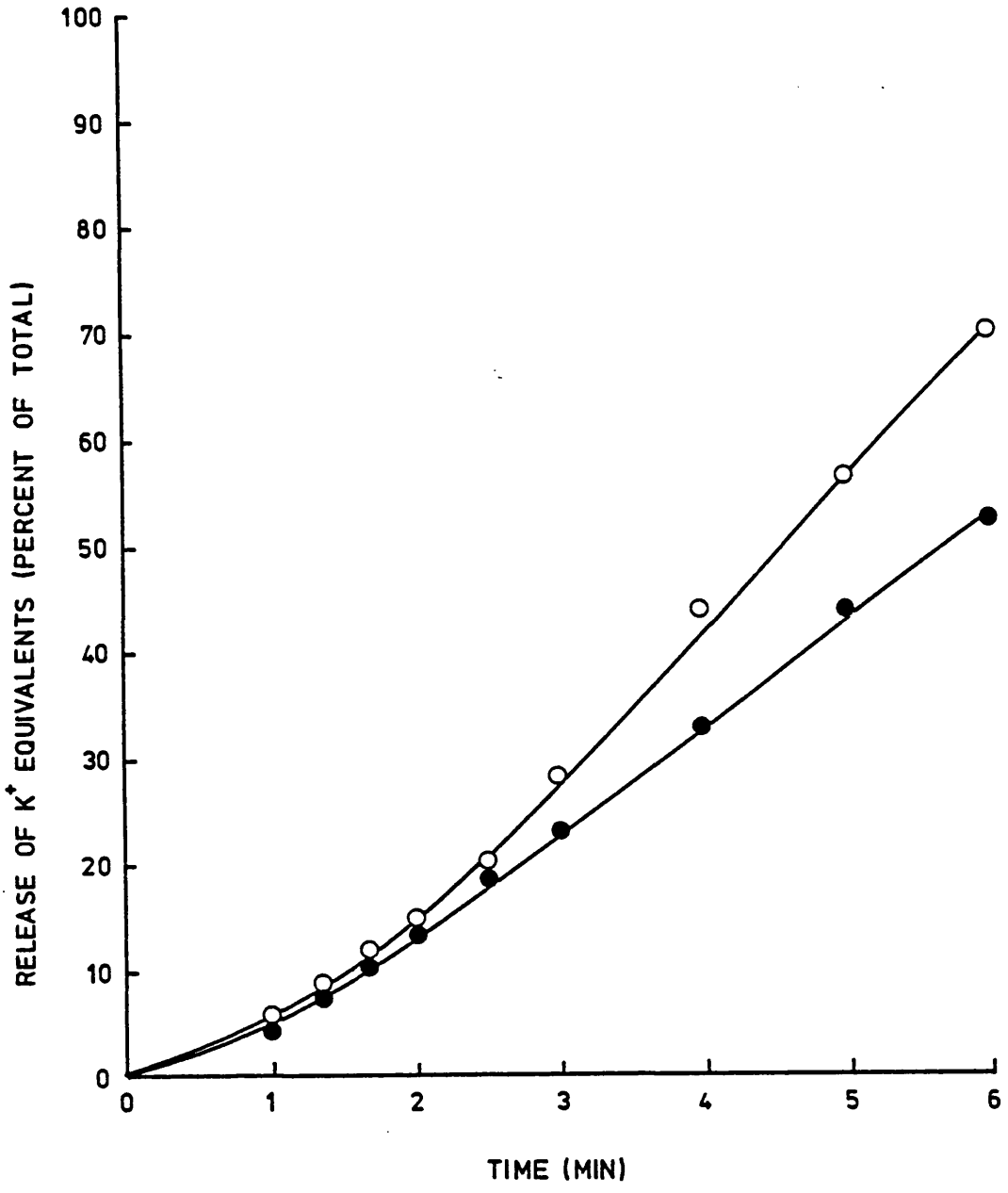


Figure 15. Time-course of release of K^+ equivalents from *Saccharomyces cerevisiae* NCYC 366 with plasma membranes enriched in PC (●) or PE (○) suspended in buffered 1.2 M-sorbitol (pH 6.0) containing 1.0 mM-SDS. The 95% confidence limit on the curve describing release from PC-enriched cells is ±0.78% and from PE-enriched cells ±3.51%. Values plotted are the average of three determinations.

Sphaeroplasts from cells with plasma membranes enriched in PC or PE were suspended in buffered 1.2 M-sorbitol supplemented with a surface-active agent, released cations at the same rate whether in the presence of CTAB (0.05 or 0.075 mM), or 0.25 or 0.50 mM-Triton X-100 (Figs. 16 and 17). However, when suspensions of PC-enriched sphaeroplasts were supplemented with 1.0 mM-SDS, they released cations at a much greater rate than those enriched in PE (Fig. 18). Even after 6 min, when populations of sphaeroplasts from cells enriched with PC lost vitually all their K^+ equivalents, populations derived from cells enriched in PE retained almost 20% of their K^+ equivalents. CTAB was the most effective in causing release of K^+ equivalents, followed by Triton X-100, whereas SDS was the least effective. There was no detectable release of cations from sphaeroplasts suspended in surfactant-free buffered sorbitol.

Liposomes. Some preliminary studies were carried out using liposomes prepared from commercially available phospholipids (Sigma). However, like Papahadjopoulos and Miller (1967) and Hsu Chen and Feingold (1973), it was found impossible to form effectively sealed liposomes using pure PE. It was possible though to produce liposomes from a mixture of PC, PE and phosphatidic acid (86, 10, 4%, w/w/w), as well as ones consisting of PC and phosphatidic acid (96, 4%, w/w). Both types of liposome released K^+ at similar rates when suspended in a range of concentrations of SDS and Triton X-100 but, when challenged with a range of concentrations of CTAB, liposomes consisting of PE, PC and phosphatidic acid released K^+ faster than those consisting of PC and phosphatidic acid.

Stable liposomes were prepared from mixtures of phospholipids extracted from cells enriched in either PC or PE, and these leaked K^+ at the same rate when suspensions were supplemented with 0.50 or 0.75 mM-

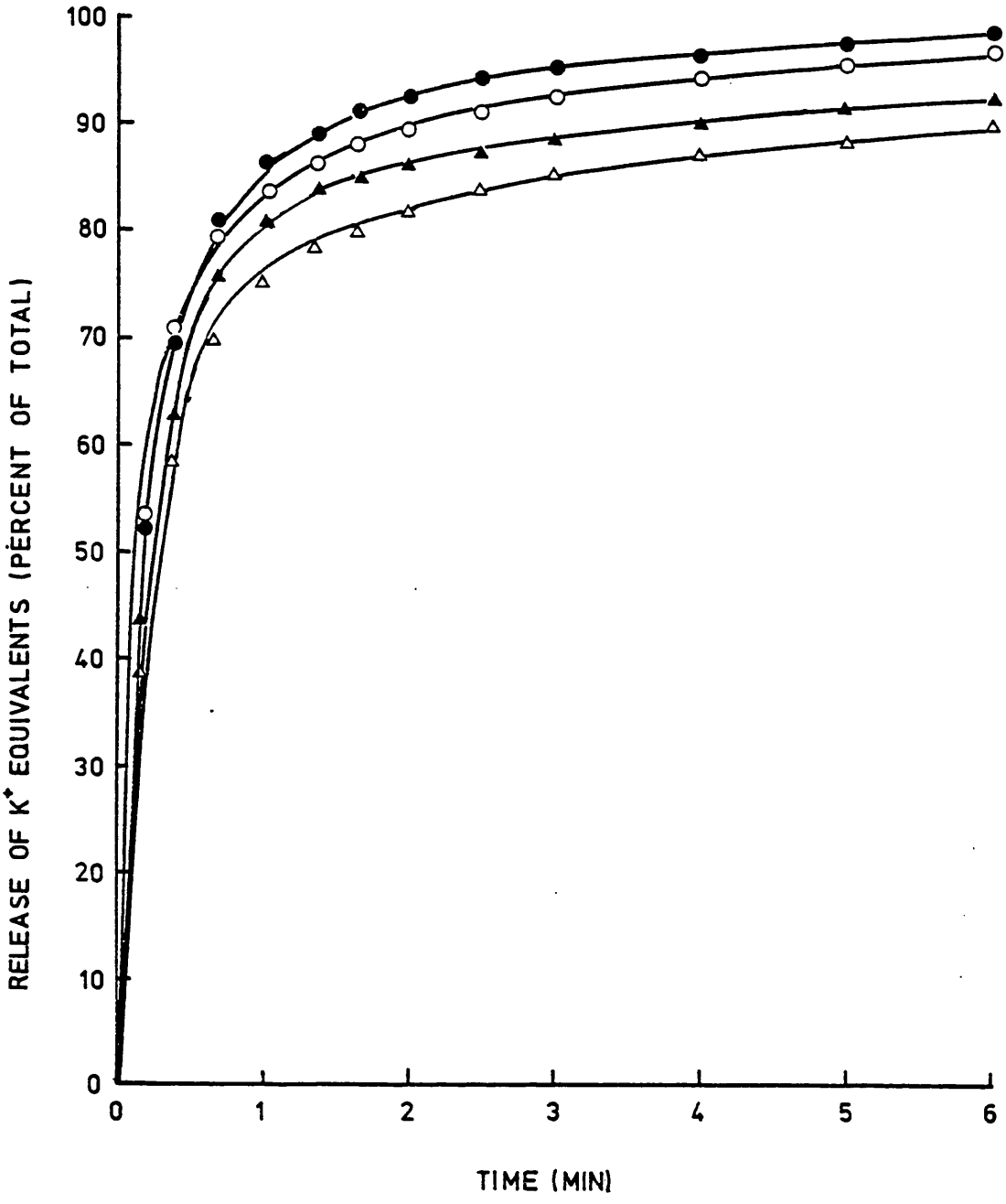


Figure 16. Time-course of release of K^+ equivalents from sphaeroplasts of *Saccharomyces cerevisiae* NCYC 366 with plasma membranes enriched in PC (closed symbols) or PE (open symbols) suspended in buffered 1.2 M-sorbitol (pH 6.0) containing CTAB at 0.05 mM (triangles) or 0.075 mM (circles). The 95% confidence limits on the curves describing release are ±1.98% (△), ±2.05% (▲), ±1.54% (○) and ±2.96% (●). Values plotted are the average of three determinations.

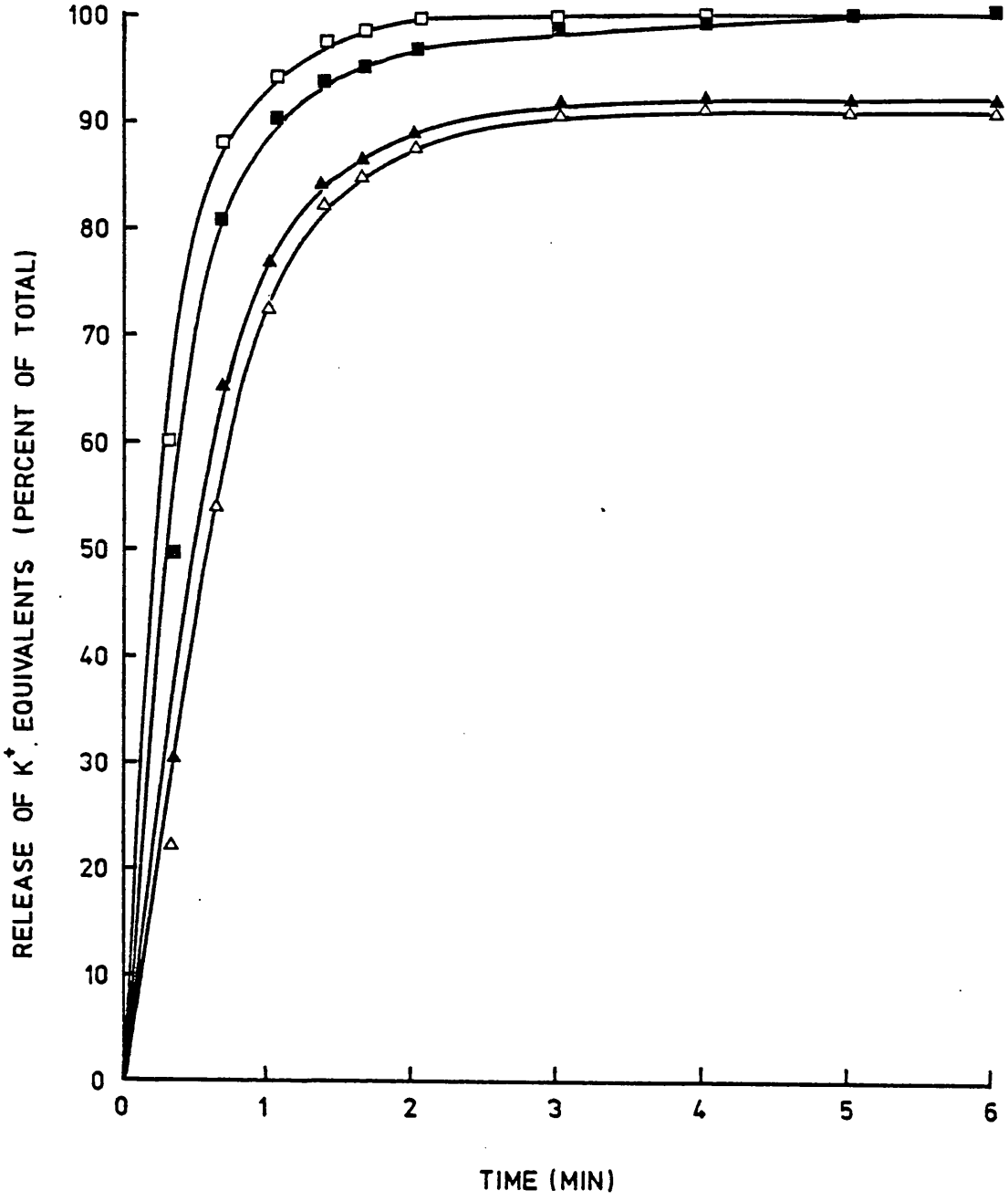


Figure 17. Time-course of release of K^+ equivalents from sphaeroplasts of *Saccharomyces cerevisiae* NCYC 366 with plasma membranes enriched with PC (closed symbols) or PE (open symbols) suspended in buffered 1.2 M-sorbitol (pH 6.0) containing Triton X-100 at 0.25 mM (triangles) or 0.50 mM (squares). The 95% confidence limits on the curves describing release are ± 2.10% (△), ± 2.30% (▲), ± 4.41% (○) and ± 2.09% (●). Values plotted are the average of three determinations.

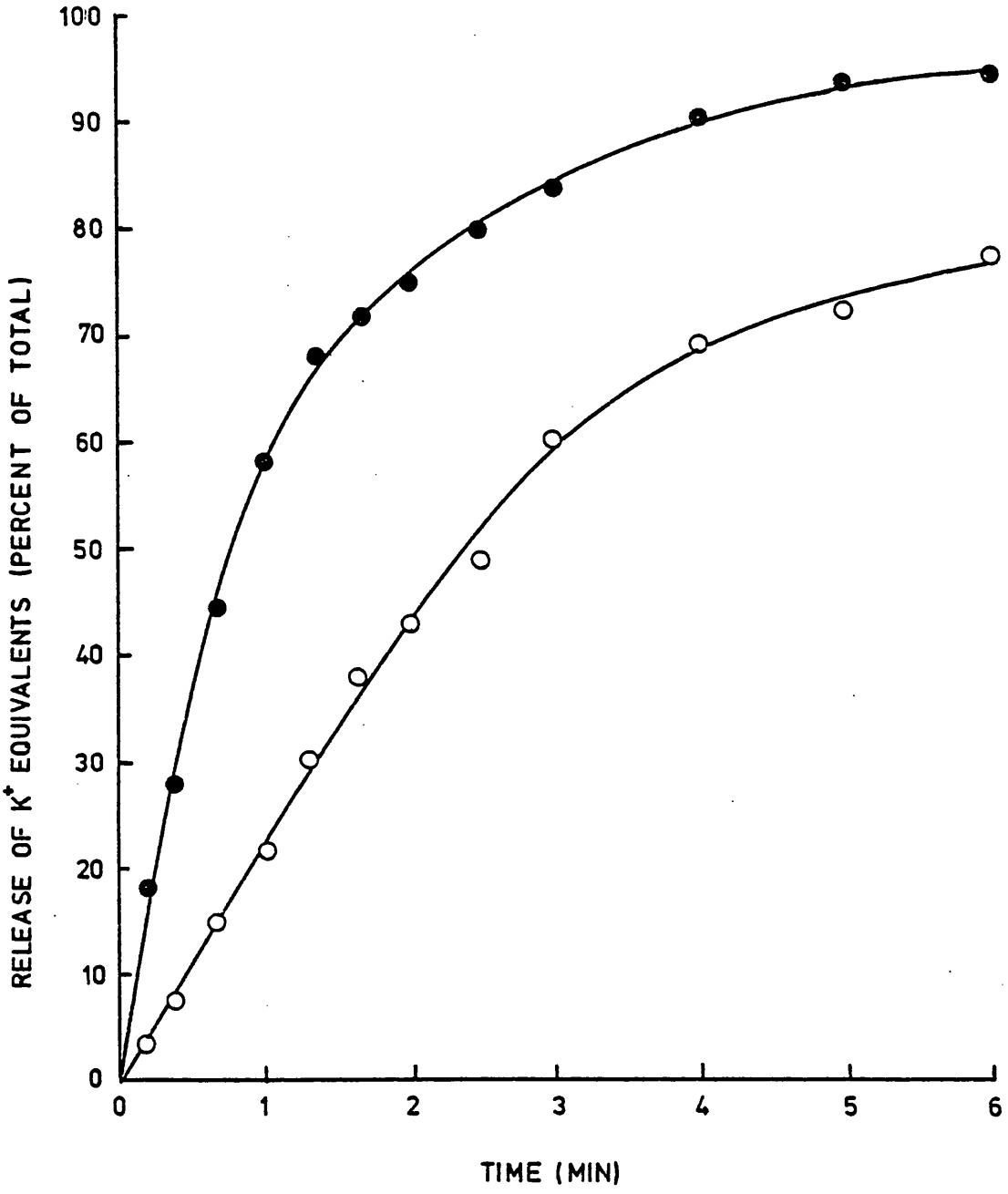


Figure 18. Time-course of release of K^+ equivalents from sphaeroplasts of *Saccharomyces cerevisiae* NCYC 366 with plasma membranes enriched in PC (●) or PE (○) suspended in buffered 1.2 M-sorbitol (pH 6.0) containing 1.0 mM-SDS. The 95% confidence limit on the curve describing release from PC-enriched sphaeroplasts is ±3.63% and from PE-enriched sphaeroplasts is ± 3.12%. Values plotted are the average of three determinations.

SDS (Fig. 19) or with 0.10 or 0.25 mM-Triton X-100 (Fig. 20). Despite persistent efforts it was not possible to record the release of K^+ from liposomes when they were suspended in a number of different concentrations of CTAB, even though there was obvious reduction in turbidity. Liposomes suspended in surfactant-free buffer did not release detectable amounts of K^+.

Properties of Cell Walls of *Saccharomyces cerevisiae* NCYC 366

In order to establish that differences in susceptibility of cells enriched in PC or PE, to SDS were not due to alterations in the structure or composition of the cell wall, an examination was made of certain wall properties.

pH-Electrophoretic Mobility. Eddy and Rudin (1958a, b) postulated the presence of three types of ionizable groups responsible for the net surface charge of yeast. Of these the phosphodiester groups of phosphomannan, which have a pK value of just less than 2, carry a negative charge over a wide range of acidities. A measure of the contribution of phosphodiester groups is given by the mobility at pH 4.0 ($m_{4.0}$). Carboxyl groups on the acidic amino-acid residues in the protein component of the cell wall were found to be only ionized above pH 4.0,whereas below pH 4.0 any decrease in net negative charge could be attributed to ionization of positively charged protonated amino groups. A measure of mobility due to protein in the wall surface is the difference between the mobilities at pH 7.0 and 3.0 ($m_{7.0} - m_{3.0}$).Therefore, from a pH mobility curve information regarding the composition of the wall surface can be obtained.

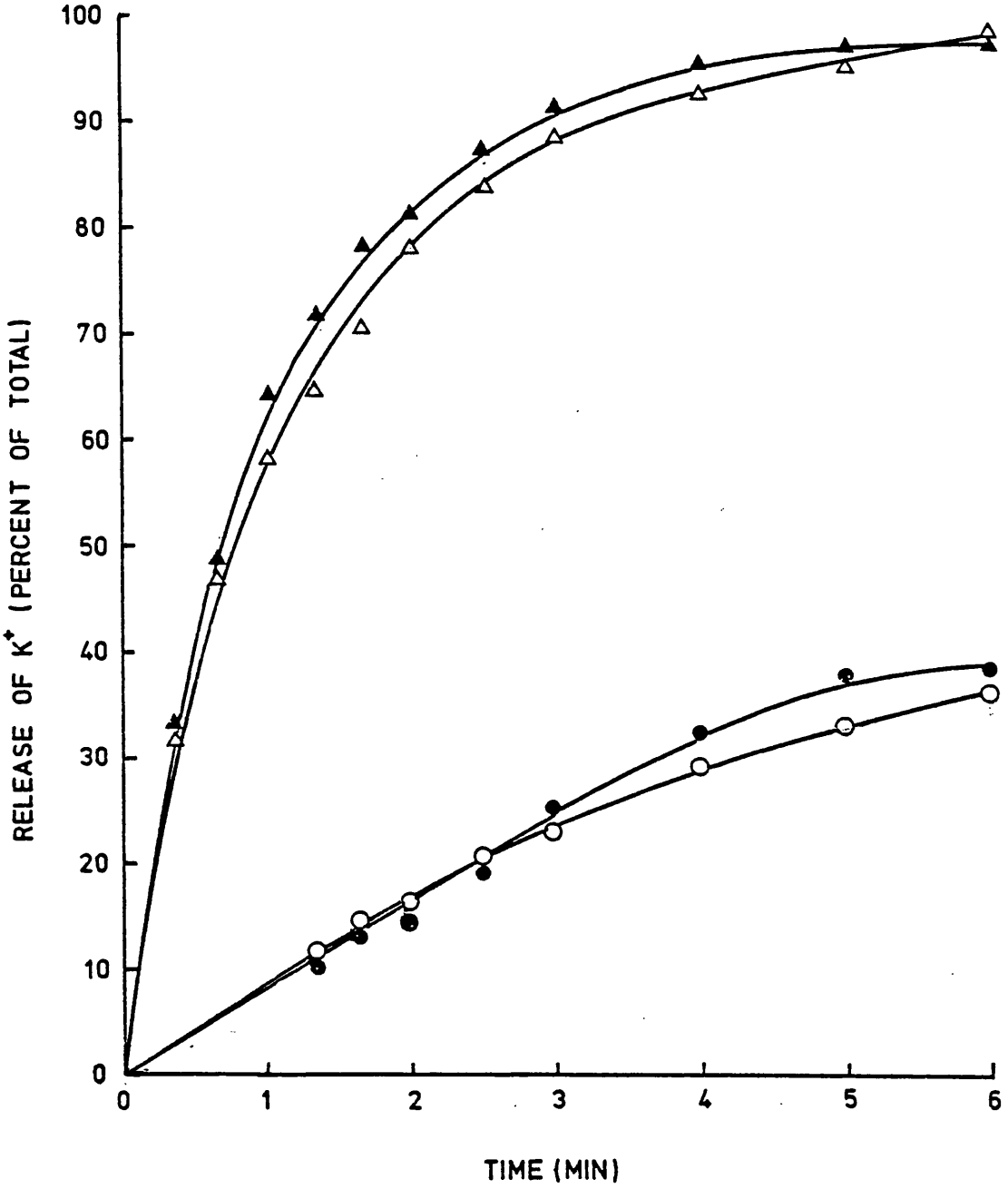


Figure 19. Time-course of SDS-induced release of K^+ from liposomes prepared from mixtures of phospholipids extracted from *Saccharomyces cerevisiae* NCYC 366 enriched with PC (closed symbols) or PE (open symbols). Release was induced by either 0.50 mM-SDS (circles) or 0.75 mM-SDS (triangles). The 95% confidence limits on the curves describing release are ± 3.41% (△), ± 2.40% (▲), ± 4.72% (○) and ± 4.51% (●). Values plotted are the average of three determinations.

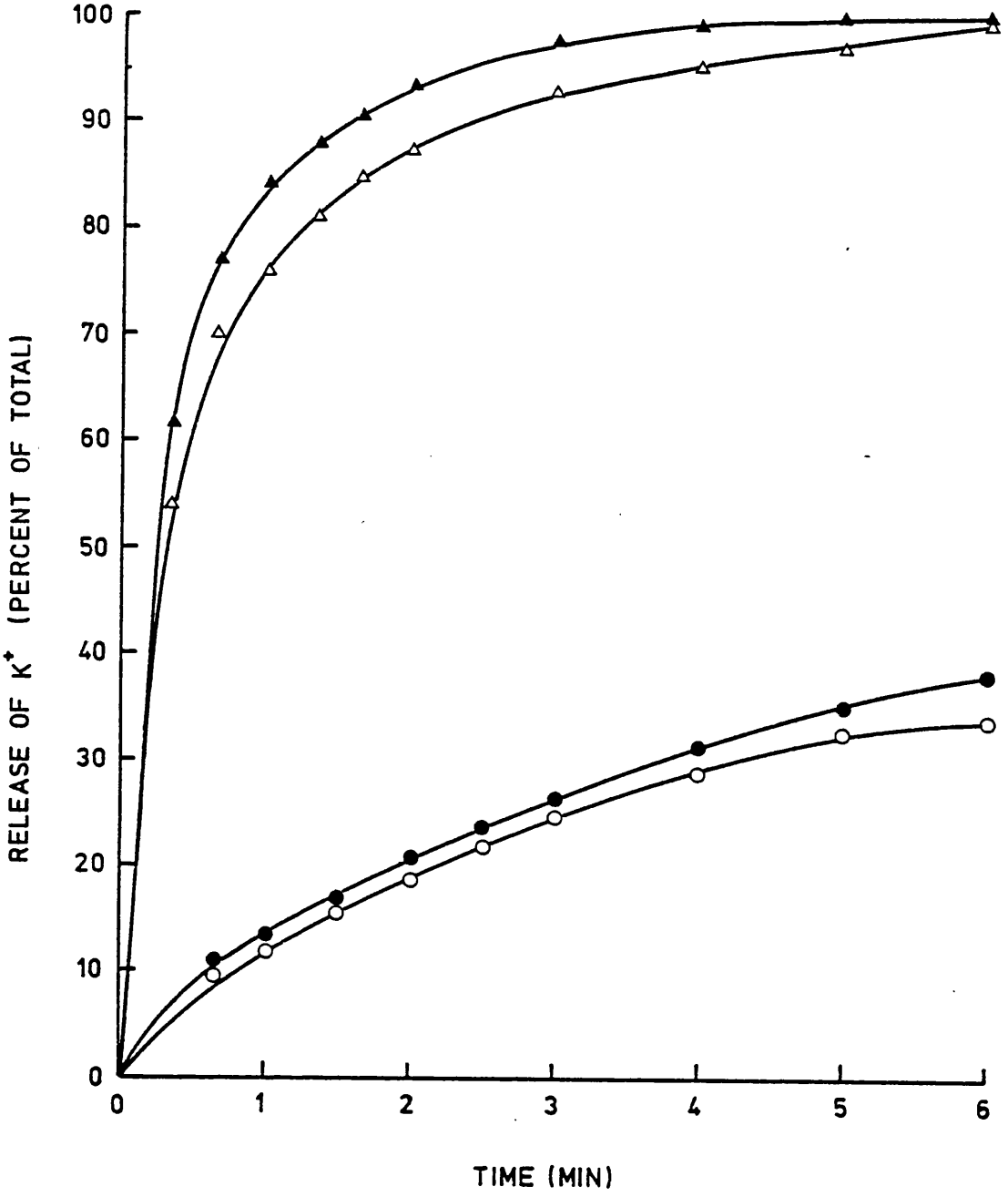


Figure 20. Time-course of Triton X-100-induced release of K^+ from liposomes prepared from mixtures of phospholipids extracted from *Saccharomyces cerevisiae* NCYC 366 enriched in PC (closed symbols) or PE (open symbols). Release was induced by either 0.10 mM-Triton X-100 (circles) or 0.25 mM - Triton X-100 (triangles). The 95% confidence limits on the curves describing release are ±4.98% (△), ±5.48% (▲), ±3.28% (○) and ±6.21% (●). Values plotted are the average of three determinations.

Furthermore, the depth of the wall being examined is known following the work of Fisher (1975) who reported that,with a buffer of ionic strength 0.05, the thickness of the surface layer in which the ionizable groups are situated is approximately 1.4 nm.

Cells enriched with PC or PE had mobilities due to phosphodiester linkages ($m_{4.0}$) of 0.945 and 0.907 $10^{-8} m^2 V^{-1} s^{-1}$ respectively (Fig. 21). There was no apparent contribution, in either type of enriched cell, from protein ($m_{7.0} - m_{3.0}$). The cell surfaces, therefore appeared to be very similar in composition down to a depth of 1.4 nm, with phosphodiester groups making the principal contribution at physiological pH values.

Porosity of Walls and Membranes. Plots of percentage uptake of a range of ethylene glycols and polyethylene glycols against molecular weight (Figs. 22a and b) gave virtually identical curves for cells enriched in either PC or PE. The uptake-exclusion threshold for the plasma membrane was found to correspond to a monodisperse ethylene glycol of molecular weight 145 and the uptake-exclusion threshold for the cell wall corresponded to a polydisperse polyethylene glycol of number-average molecular weight 650. There was a greater scatter of points on the PC curve, although this was most likely due to inaccuracies in measuring very small uptakes of solutes.

Susceptibility to β-glucanase. Cells enriched in PC or PE were converted into sphaeroplasts by digestion with Zymolyase-5000 which was used as a commercial source of β-glucanase (Fig. 23). In all three concentrations

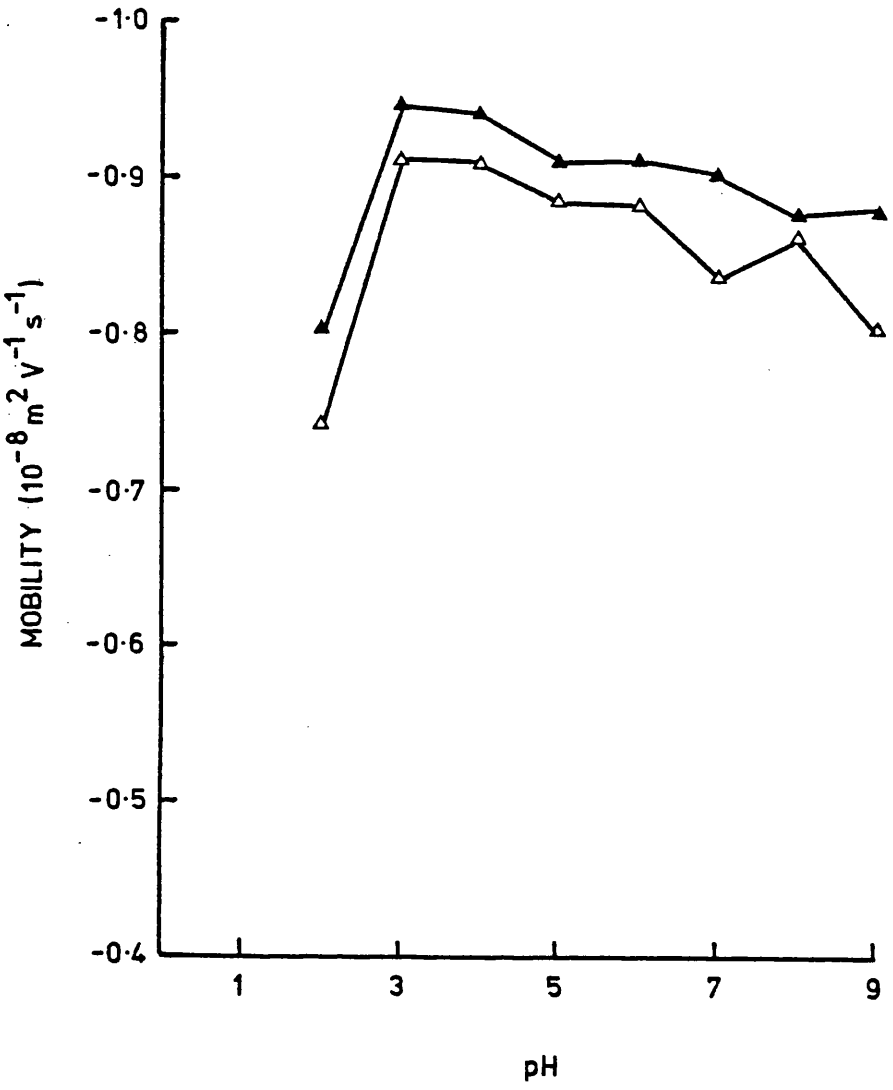
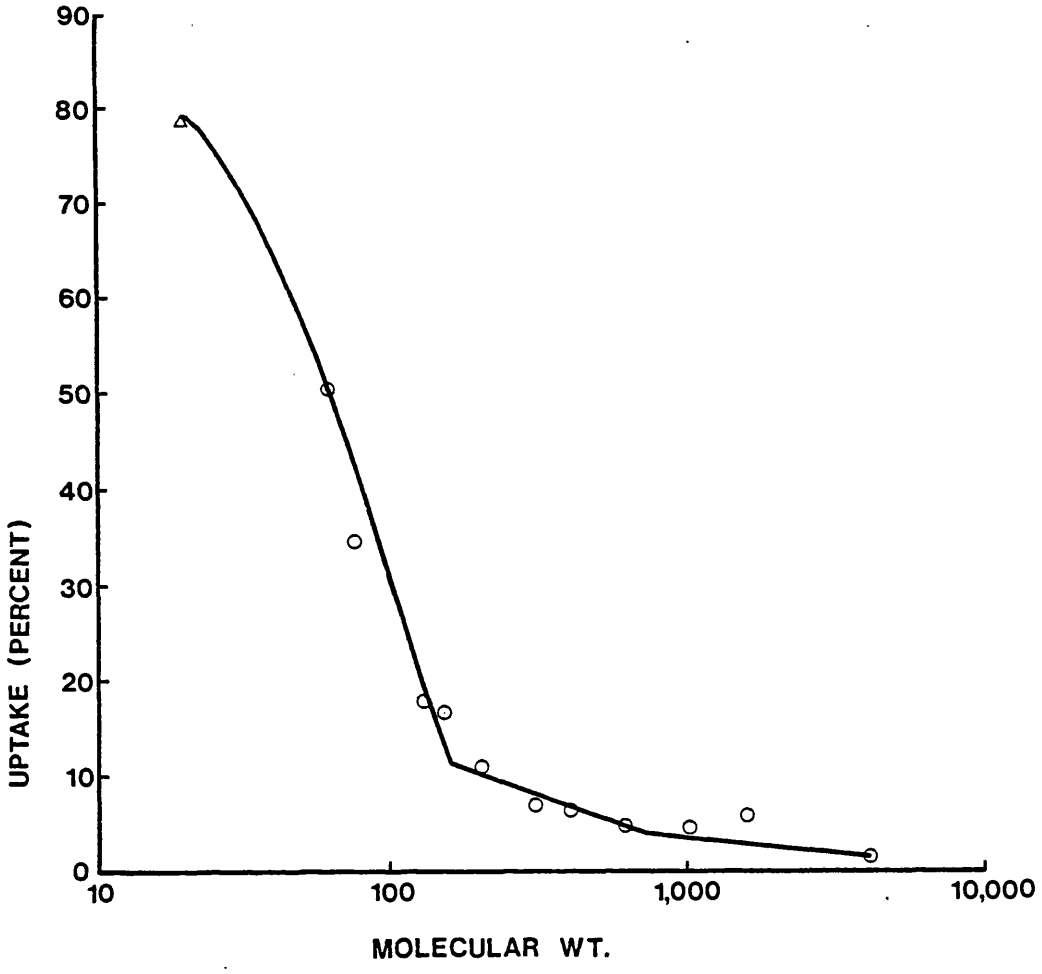


Figure 21. pH-Electrophoretic mobility curves of *Saccharomyces cerevisiae* NCYC 366 enriched in either PC (▲) or PE (△) at an ionic strength of 0.05. Values plotted are the means of 20 observations and the standard error of the mean was less than 2%.

Figures 22a and b. Permeability of *Saccharomyces cerevisiae* NCYC 366 enriched with PE (a, open symbols) or PC (b, closed symbols) to a series of probing molecules as a function of the logarithm of the molecular weight. The molecular weights or number-average molecular weights of the probing molecules used (indicated by circles) were 62.1 (4, 3), 76.1 (3, 4), 134.2 (4, 4), 150.2 (3, 4), 200 (4, 3), 300 (4, 3), 400 (4, 4), 600 (7, 6), 1000 (4, 5), 1540 (3, 4) and 4000 (8, 5). The numbers in parentheses indicate the number of independent determinations carried out with cells enriched in PE or PC respectively. Triangles indicate the percentage uptake of tritiated water, both values being the average of four determinations.


90
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UPTAKE (PERCENT)
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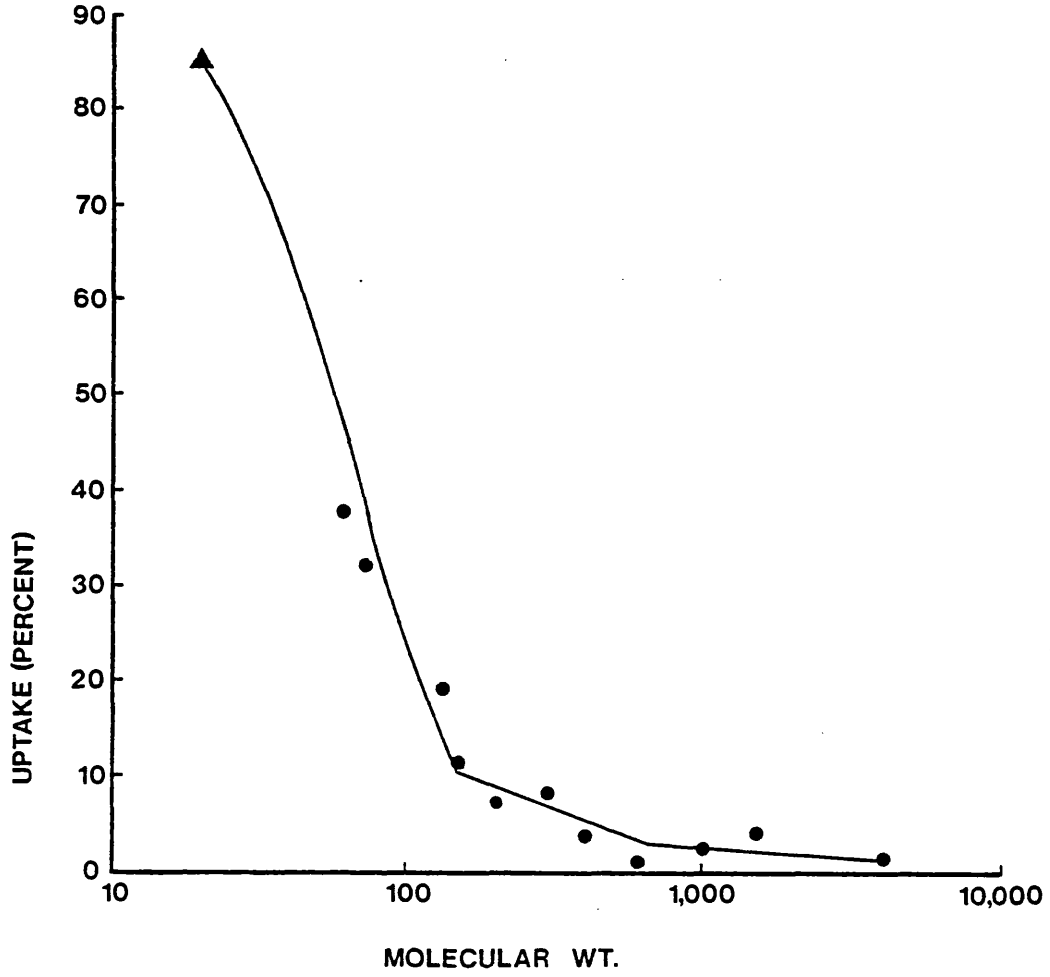
MOLECULAR WT.


90
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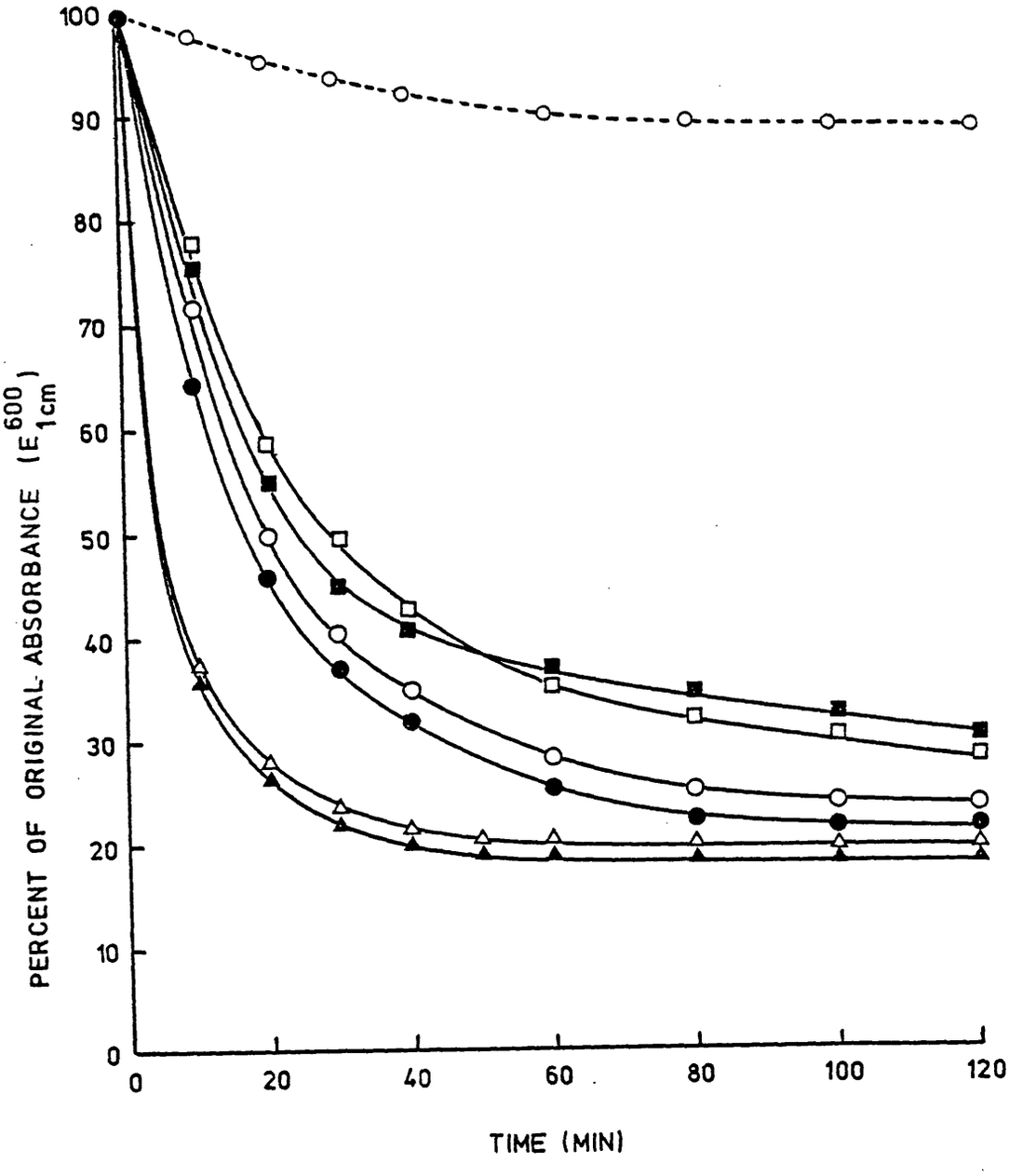


Figure 23. Time-course of the formation of sphaeroplasts from *Saccharomyces cerevisiae* NCYC 366 either enriched in PC (closed symbols) or PE (open symbols). Zymolyase-5000 concentrations were 0.1 mg (squares), 0.2 mg (circles) and 0.4 mg (triangles) per 6 mg dry wt. of cells. The broken line (O) shows a typical curve after dilution of the suspension (0.1 ml) in buffer (2.9 ml), whilst the continuous lines represent suspensions similarly diluted in water. Values plotted are the average of two determinations.

of the enzyme preparation used (0.1, 0.2 and 0.4 mg/6mg dry wt. cells) the two types of enriched cells became osmotically sensitive at the same rate. This suggested that there was no difference in the content or structure of β-glucans in cell walls.

Analysis of Cell Walls. It can be seen from carbohydrate and protein analyses (Table 2), that both types of enriched cell walls contained virtually the same amounts of glucan, mannan and protein. The remaining 2.5 to 3.5% of the wall material not accounted for was most likely composed of chitin and phosphorus. Therefore, within the limitations of a gross chemical analysis, walls from PC and from PE-enriched cells appear to have a similar composition.

Effect of SDS on the Ultrastructure of *Saccharomyces cerevisiae* NCYC 366

Transmission electron micrographs of thin sections through cells showed that both types of enriched cell were spherical with a conspicuous vacuole (Plates 1 and 2). In the cytoplasm there were various vesicles and several mitochondria, but nuclei could not be positively identified, which was undoubtedly due to difficulties in fixing and staining of yeast cells. On a closer examination of the cell envelope, it was possible to see the plasma membrane as a trilaminar structure, which formed invaginations into the cytoplasm at intervals (Plate 3). After examination of a large number of sections, it was apparent that there were no differences between the ultrastructure of cells with plasma membranes enriched in PC and those with plasma membranes enriched with PE.

Table 2. Composition of walls of *Saccharomyces cerevisiae* enriched in PC or PE

wall component	Content (mg/100 mg dry wt.) of walls from cells enriched in: PC	PE
Glucan	35.9 ± 8.9 (5)	37.2 ± 7.2 (5)
Mannan	48.0 ± 8.7 (5)	48.3 ± 5.2 (5)
Protein	12.5 ± 1.7 (4)	12.1 ± 5.3 (4)

Values quoted are means ± 95% confidence limits. The number of independent determinations carried out is indicated in parentheses beside each analytical value.

There was a marked change in ultrastructure of cells enriched in PC or PE following their suspension in 67 mM-KH_2PO_4 buffer (pH 4.5) supplemented with 0.10 mM-SDS for 30 min. In both types of enriched cells, the vacuoles were severely disrupted. There was also a complete absence of vesicles and little evidence of mitochondria (Plate 4). Closer examination of the cell envelope showed that the plasma membrane was, as far as could be seen, still intact although the characteristic invaginations had disappeared (Plates 5 and 6). There was no apparent difference in response of the two types of enriched cells to SDS.

AN ELECTRON-MICROSCOPE STUDY OF CELLS AND FORMATION OF SPHAEROPLASTS OF *SACCHAROMYCES CEREVISIAE* NCYC 366

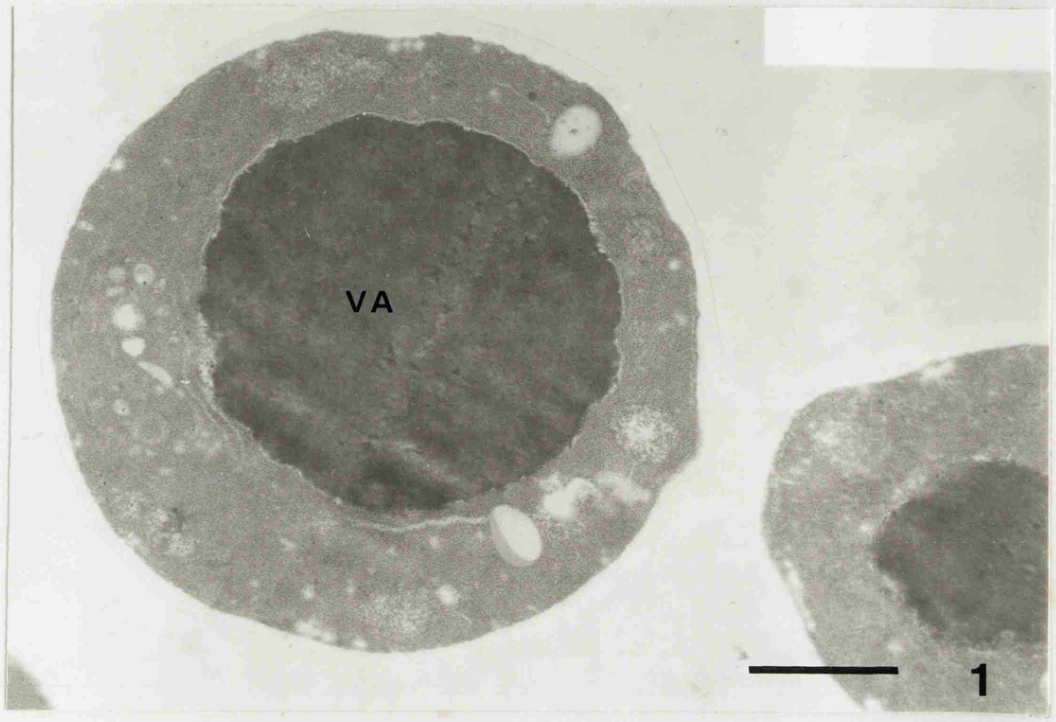
In an attempt to discover why the sensitivities of PC-or PE-enriched cells to SDS were reversed in sphaeroplasts prepared from these two types of enriched cells, an electron microscope study was undertaken. It was hoped that the different sensitivities might be explained by differences in morphology or by changes occurring during the sphaeroplasting process.

Cells and Sphaeroplasts of *Saccharomyces cerevisiae* NCYC 366

Examination with the scanning microscope (Plates 7 and 8) revealed no difference in shape, size or surface appearance between cells enriched in PC and those enriched in PE. Both populations of cells appeared almost spherical, with one or two buds and sometimes in chains of two or three. The surface had a slightly wavy appearance.

Plate 1. Section through *Saccharomyces cerevisiae* NCYC 366 enriched in PC. The vacuole (VA) occupies the central region with a number of vesicles and mitochondria in the cytoplasm. The cell is surrounded by an electron-transparent cell wall which forms an attachment to a bud. Bar marker represents 1 μm.

Plate 2. Section through *Saccharomyces cerevisiae* NCYC 366 enriched in PE. The vacuole (VA), which occupies the central region, is fragmented. There are a number of vesicles and mitochondria in the surrounding cytoplasm. An electron-transparent cell wall surrounds the cell. Bar marker represents 1 μm.


VA
1

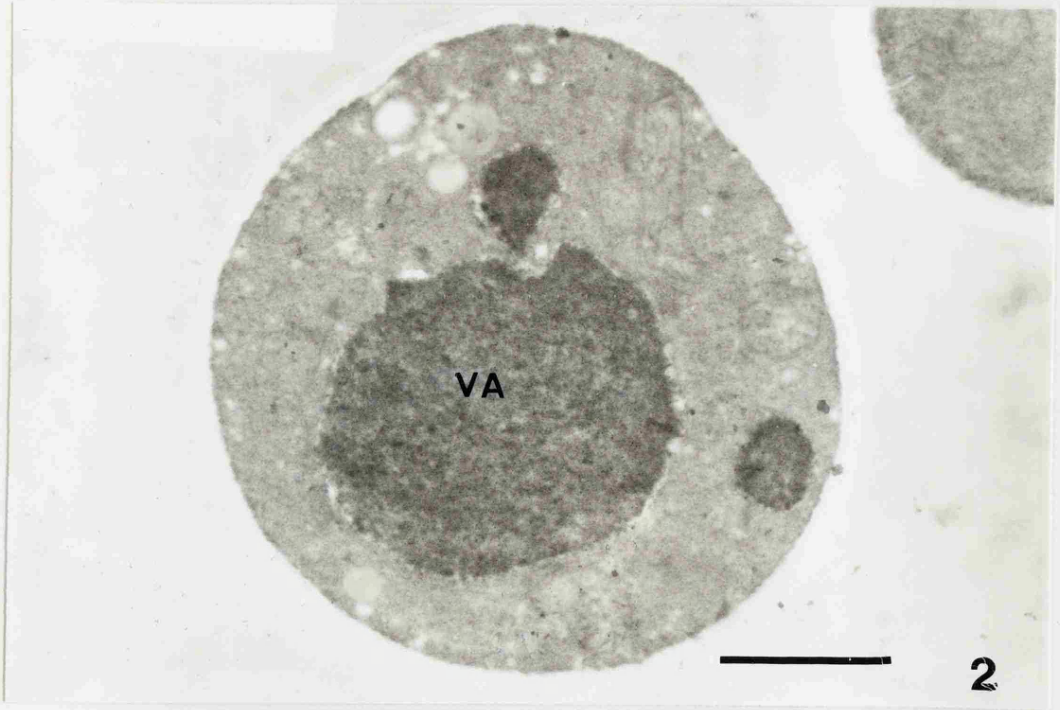
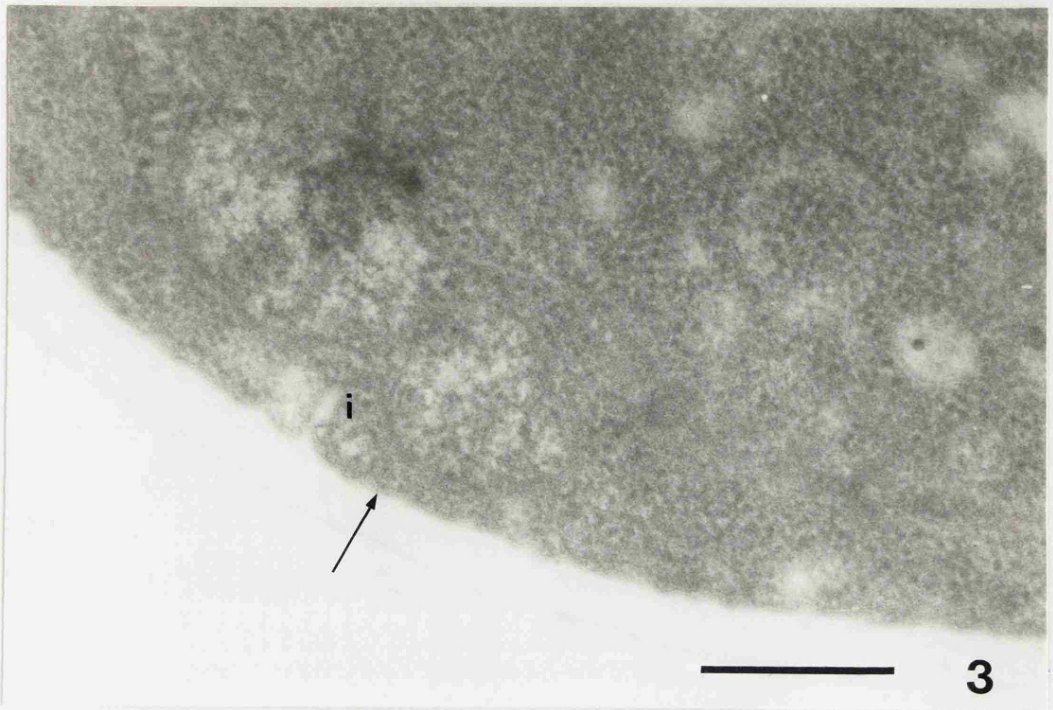

VA
2

Plate 3. Section through part of *Saccharomyces cerevisiae* NCYC 366 enriched in PC. The cytoplasm is surrounded by the trilaminar plasma membranes (arrow) which is invaginated (i) and exterior to this lies an electron-transparent cell wall. Bar marker represents 0.25 μm.

Plate 4. Section through *Saccharomyces cerevisiae* NCYC 366 enriched in PC after suspension in phosphate buffer (pH 4.5) containing 0.10 mM-SDS for 30 min. The vacuole (VA) is disrupted and most of the cytoplasmic inclusions have disappeared. The cell is attached to the mother cell by an electron-transparent cell wall. Bar marker represents 1 μm.



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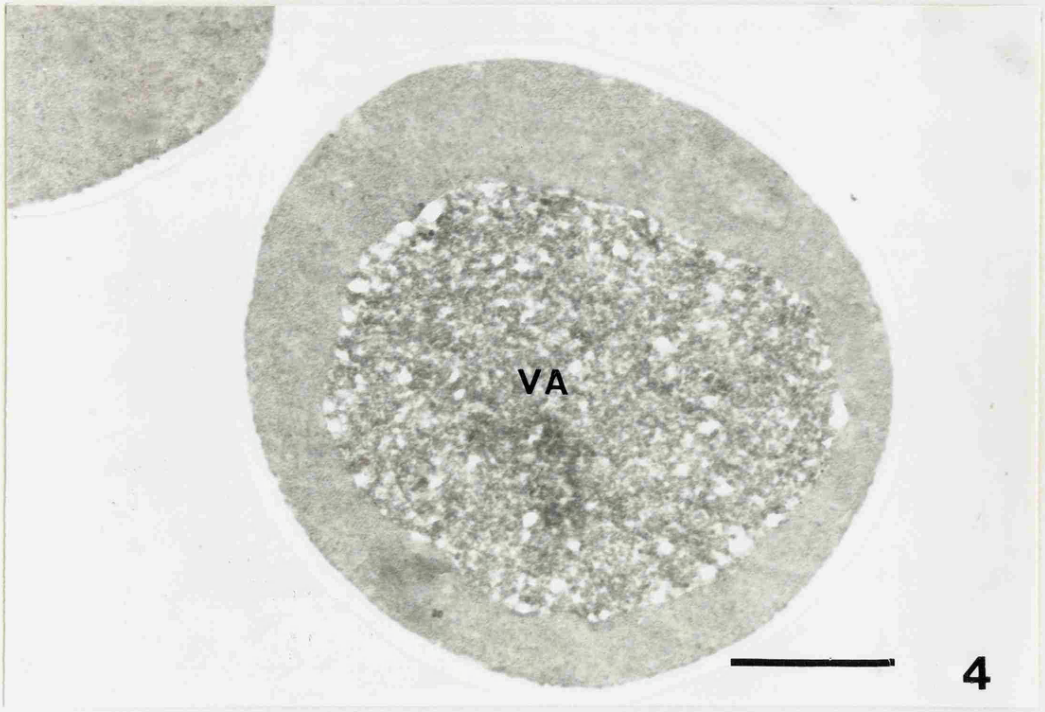

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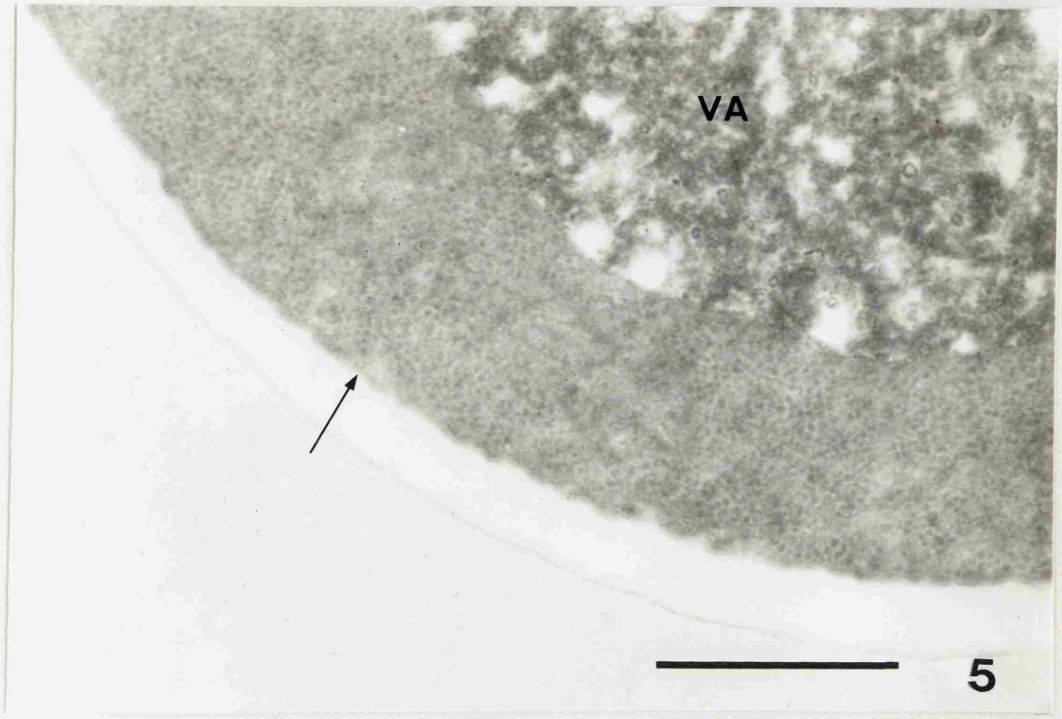
Plate 5. Section through part of *Saccharomyces cerevisiae* NCYC 366 enriched in PC after suspension in phosphate buffer (pH 4.5) containing 0.10 mM-SDS for 30 min. The vacuole (VA) is disrupted and the plasma membrane (arrow), although apparently intact, has no characteristic invaginations. The cell is surrounded by an electron-transparent cell wall. Bar marker represents 0.5 μm.

Plate 6. Section through part of *Saccharomyces cerevisiae* NCYC 366 enriched in PE after suspension in phosphate buffer (pH 4.5) containing 0.10 mM-SDS for 30 min. The vacuole (VA) is disrupted and the plasma membrane (arrow), although apparently intact, has no characteristic invaginations. The cell is surrounded by an electron-transparent cell wall. Bar marker represents 0.5 μm.


VA
5

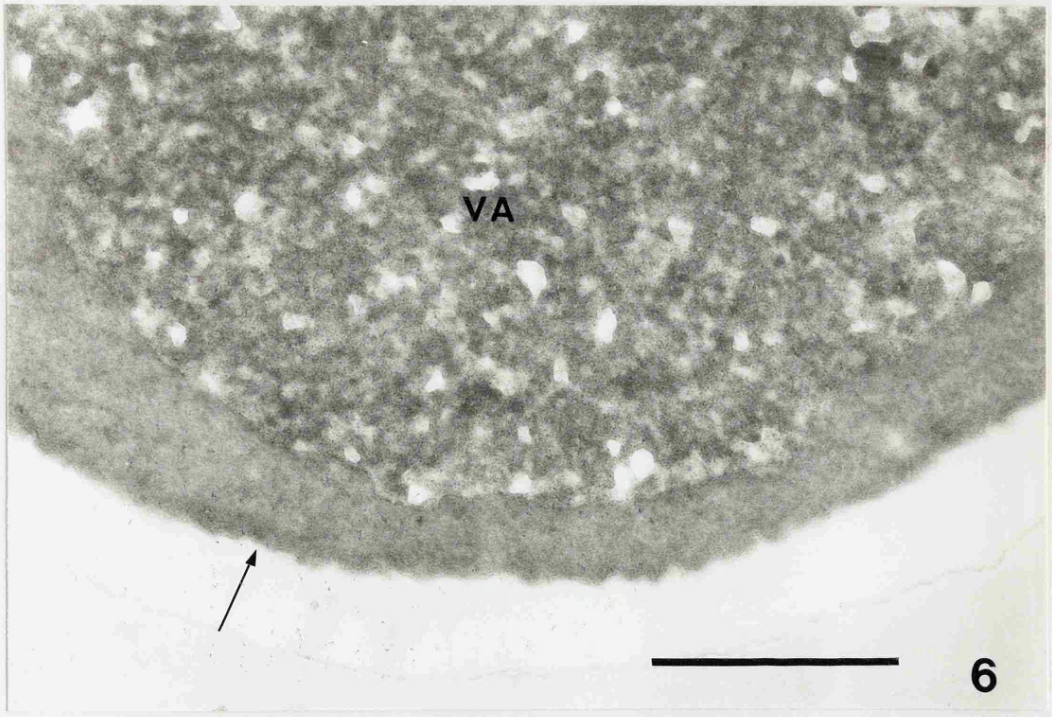
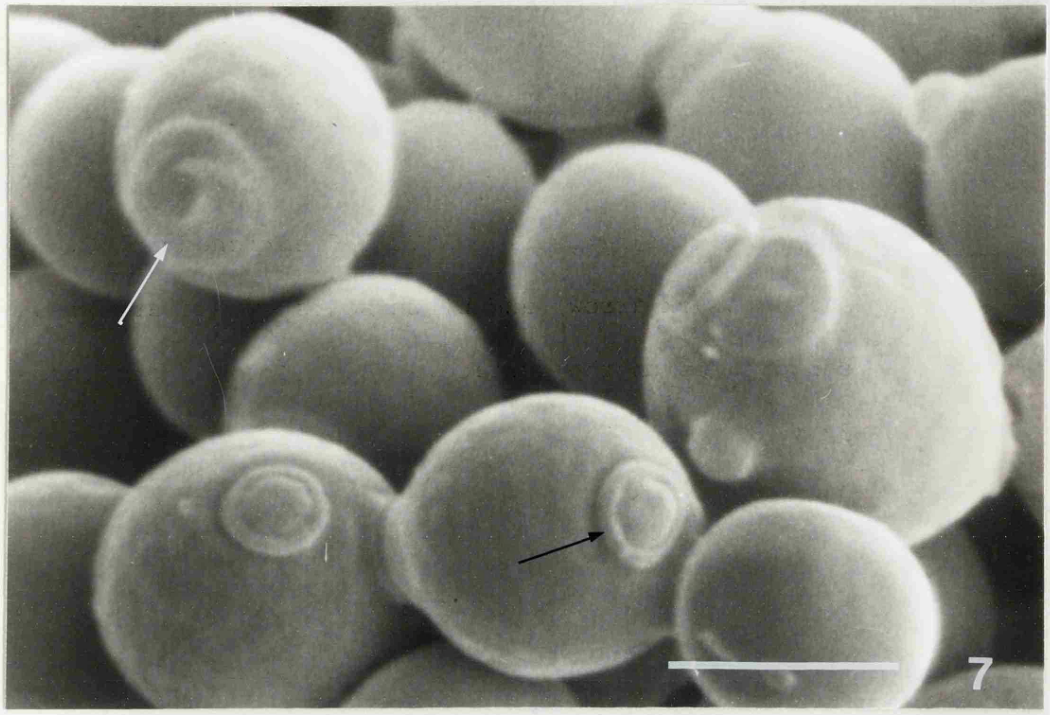

VA
6

Plate 7. *Saccharomyces cerevisiae* NCYC 366 enriched in PC. Bud scars (black arrow) and birth scars (white arrow) are conspicuous on the surface. Bar marker represents 3 µm.

Plate 8. *Saccharomyces cerevisiae* NCYC 366 enriched in PE. Bud scars (black arrow) and birth scars (white arrow) are conspicuous on the surface. Bar marker represents 3 µm.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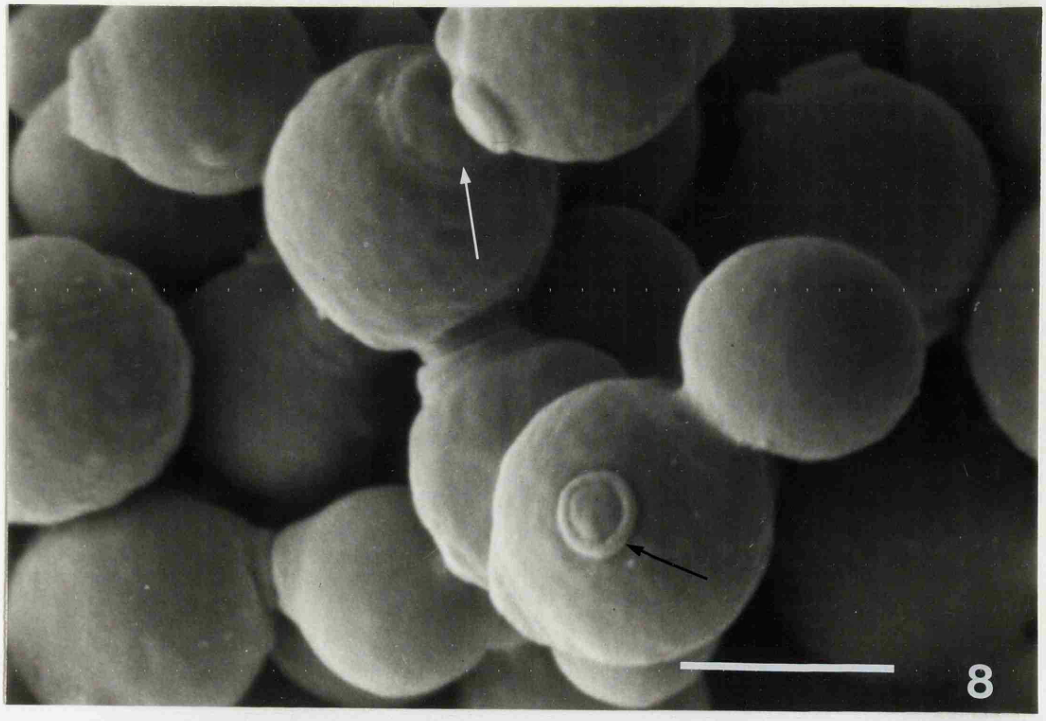
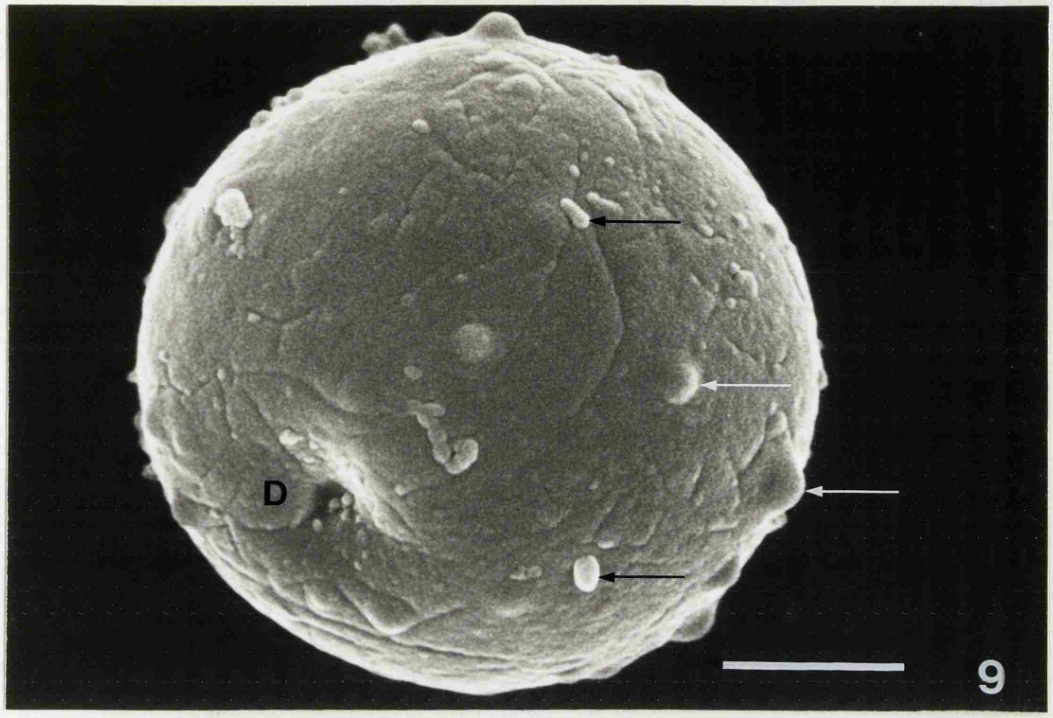

8

Although some differences were observed in populations of sphaeroplasts derived from PC- or PE-enriched cells (Plates 9, 10, 11), such as degree of invagination and amount of wall material left on the surface, these variations were present in both populations. Larger (older) cells appeared to produce larger more invaginated sphaeroplasts (Plate 11). A number of sphaeroplasts had depressions in the surface, but these were also present in both populations of sphaeroplasts. An unexpected feature of sphaeroplasts from both types of enriched cells was the number of protruberances through the surface of the plasma membrane (Plate 12) and these were apparent on most cells whatever the nature of the enrichment, to a greater or lesser extent (Plates 9, 10, 11).

Transmission electron microscopy of thin sections of sphaeroplasts from PC-or PE-enriched cells revealed that there were no differences between populations of the two types of enriched sphaeroplasts. In the cytoplasm of both types of enriched sphaeroplast vesicles of different electron densities, mitochondria, nuclei and cell vacuoles were visible, as well as various internal membranes which are sometimes seen lying parallel to the plasma membrane (Plates 13 and 14). These internal membranes were probably part of the endoplasmic reticulum. Although both electron micrographs (Plates 13 and 14) show sphaeroplasts with a large nucleus and only a small vacuole this was not significant and due to the plane of the section. The internal structure of sphaeroplasts was better preserved than previous electron micrographs of cells (Plates 1 and 2). The protruberances seen in scanning electron microscopy were caused by vesicles lying under the surface of the membrane, forcing the membrane to bulge. On a closer inspection these vesicles appeared bounded on the exterior only by the plasma

Plate 9. Sphaeroplast of *Saccharomyces cerevisiae* NCYC 366 enriched in PC. The plasma membrane is lightly invaginated and still retains some cell-wall material (black arrows). There are a number of protruberances (white arrows) on the surface as well as a depression (D). Bar marker represents 1 μm.

Plate 10. Sphaeroplast of *Saccharomyces cerevisiae* NCYC 366 enriched in PC. The deeply invaginated plasma membrane still retains some cell-wall material (black arrows). There are a number of protruberances on the surface (white arrows). Bar marker represents 1 μm.


D
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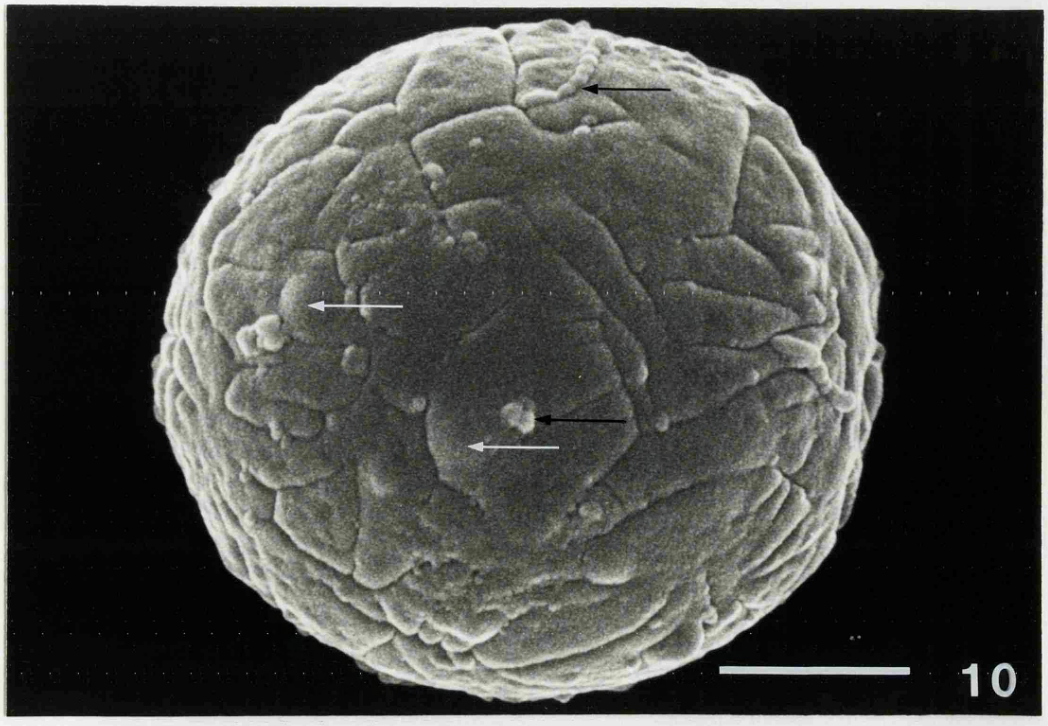
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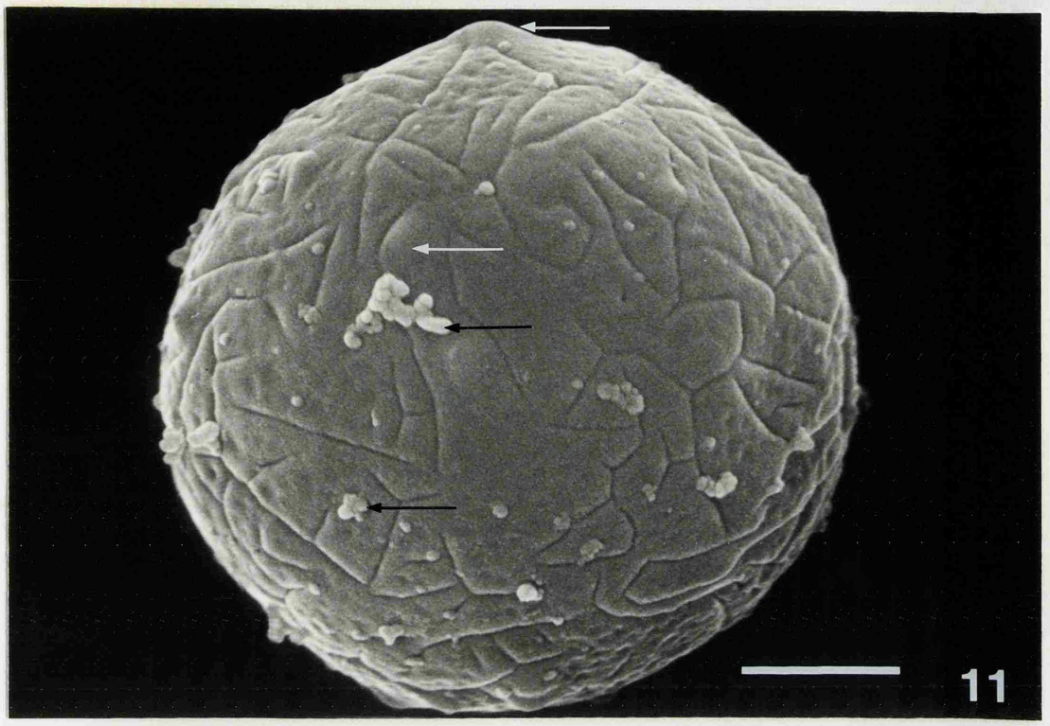
Plate 11. Sphaeroplast of *Saccharomyces cerevisiae* NCYC 366 enriched in PE. The plasma membrane has a deeply invaginated surface and still retains some cell-wall material (black arrows). There are a number of protruberances visible on the surface (white arrows). Bar marker represents 1 µm.

Plate 12. Part of surface of sphaeroplast of *Saccharomyces cerevisiae* NCYC 366 enriched in PE showing a protruberance (arrow). Bar marker represents 0.25 µm.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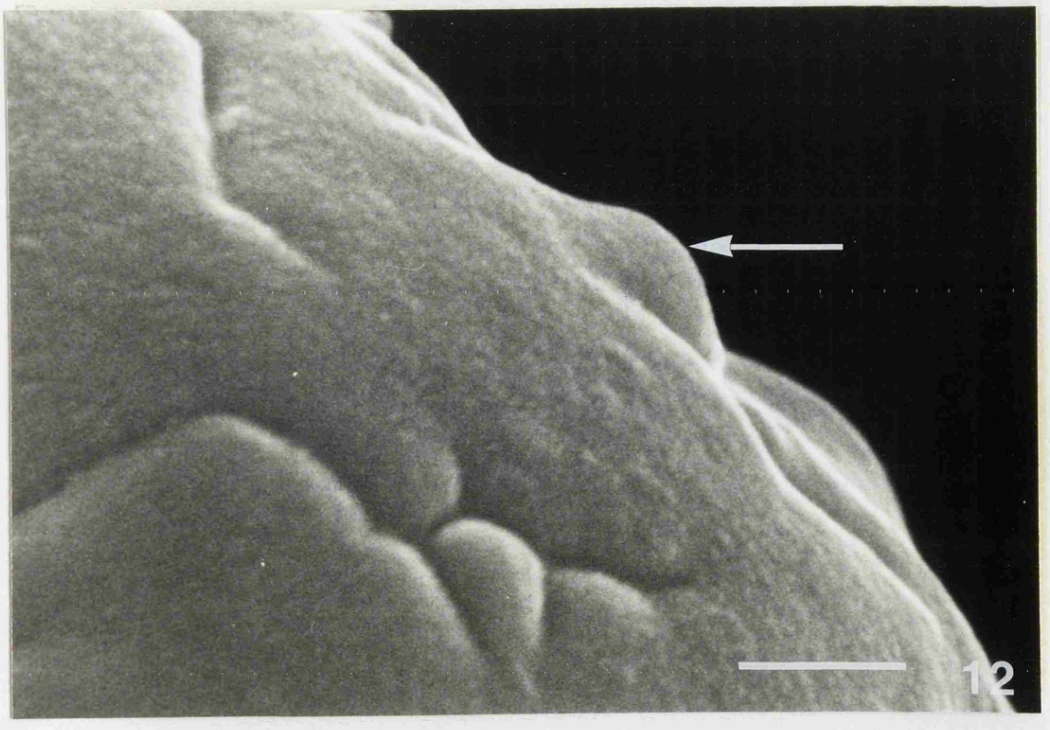
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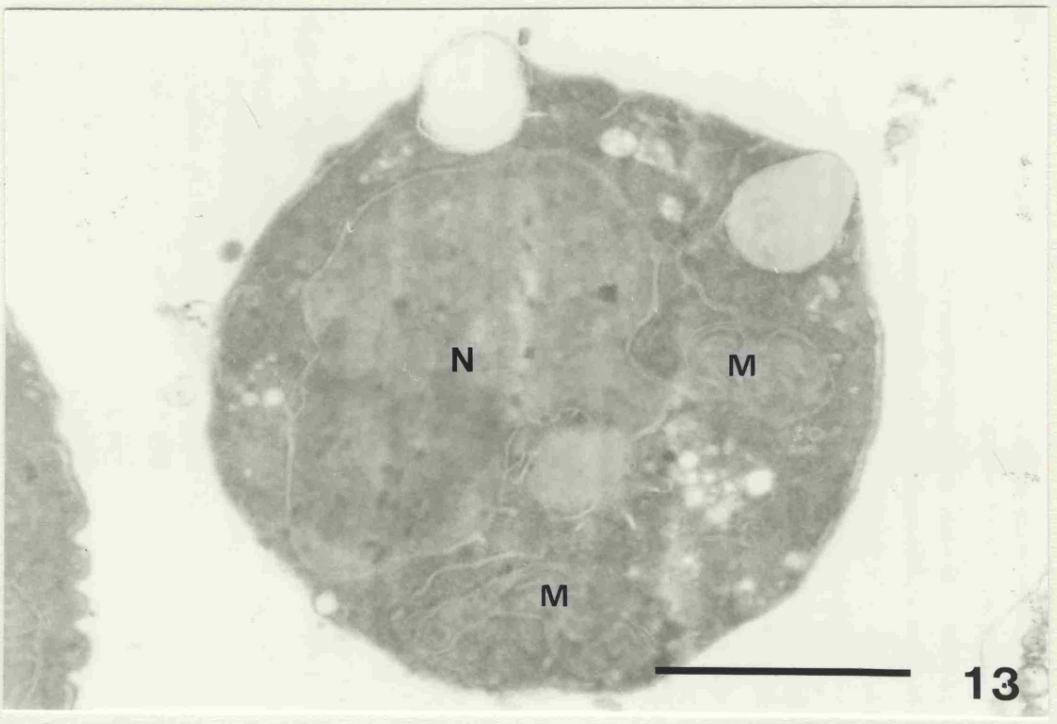
Plate 13. Tangential section through a sphaeroplast of *Saccharomyces cerevisiae* NCYC 366 enriched in PE. A large part of the nucleus (N) occupies the central region, but no vacuole is present in this section. Mitochondria (M) are visible in the cytoplasm and vesicles can be seen internally or protruding under the surface of the plasma membrane. There are many internal membranes and some appear parallel to the plasma membrane. Bar marker represents 1 μm.

Plate 14. Section through a sphaeroplast of *Saccharomyces cerevisiae* NCYC 366 enriched in PC. Centrally there is a nucleus (N) and part of the vacuole (VA). Mitochondria (M) are visible in the cytoplasm and also vesicles which can be seen internally or protruding under the surface of the plasma membrane. There are many internal membranes and some appear parallel to the plasma membrane. Bar marker represents 1 μm.


N
M
M
13

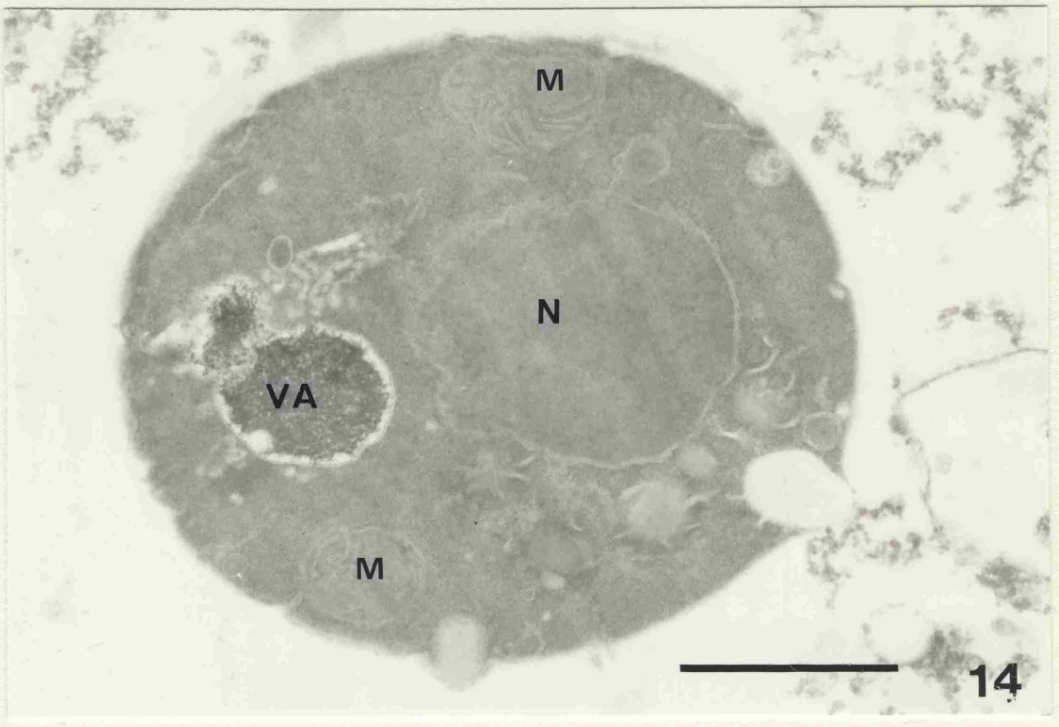
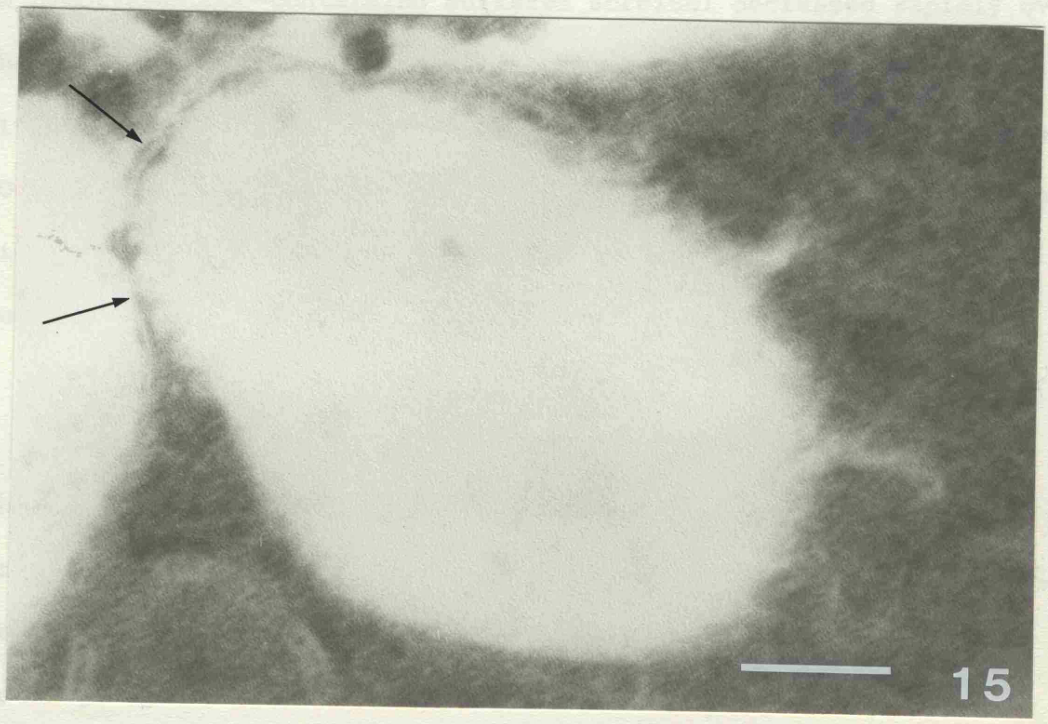

M
N
VA
M
14

Plate 15. Section through part of a sphaeroplast of *Saccharomyces cerevisiae* NCYC 366 enriched in PC showing a protruding vesicle bounded by the plasma membrane (arrow). Bar marker represents 0.1 µm.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membrane (Plate 15).

Study of the Formation of Sphaeroplasts from *Saccharomyces cerevisiae* NCYC 366

Since there was no apparent difference in the morphology of the two types of enriched cells and sphaeroplasts, unenriched cells were used in this study. The absorbance of populations of cells incubated in Zymolyase-5000-containing buffered sorbitol decreased rapidly over the first 15 min, as determined by diluting the suspension (0.1 ml) with water (2.9 ml), and maximum osmotic sensitivity was observed after 30 min (Fig. 24). There was little decrease in absorbance over the 60 min incubation period when the suspension was similarly diluted in buffer.

Cells before Zymolyase treatment (Plate 16) were spherical in shape, with bud and birth scars on the surface. Bud scars were arranged in a formation typical of a diploid strain of yeast. Cells showed a tendency to form short chains and a variety of stages of budding were visible. After as little as 5 min incubation with Zymolyase cells were greatly changed in morphology, becoming irregular in shape and having quantities of partially digested material attached to them (Plate 17). One or two sphaeroplasts were seen at this stage but these were smaller than normal cells and elongated, suggesting that they were buds. After 20 min incubation the structures still appeared irregular, but with a marked tendency to stick together (Plate 18). Only about 20% of the population had been completely converted into sphaeroplasts. After 30 min incubation, a time which coincided with maximum osmotic sensitivity, only approximately 25% of the structures

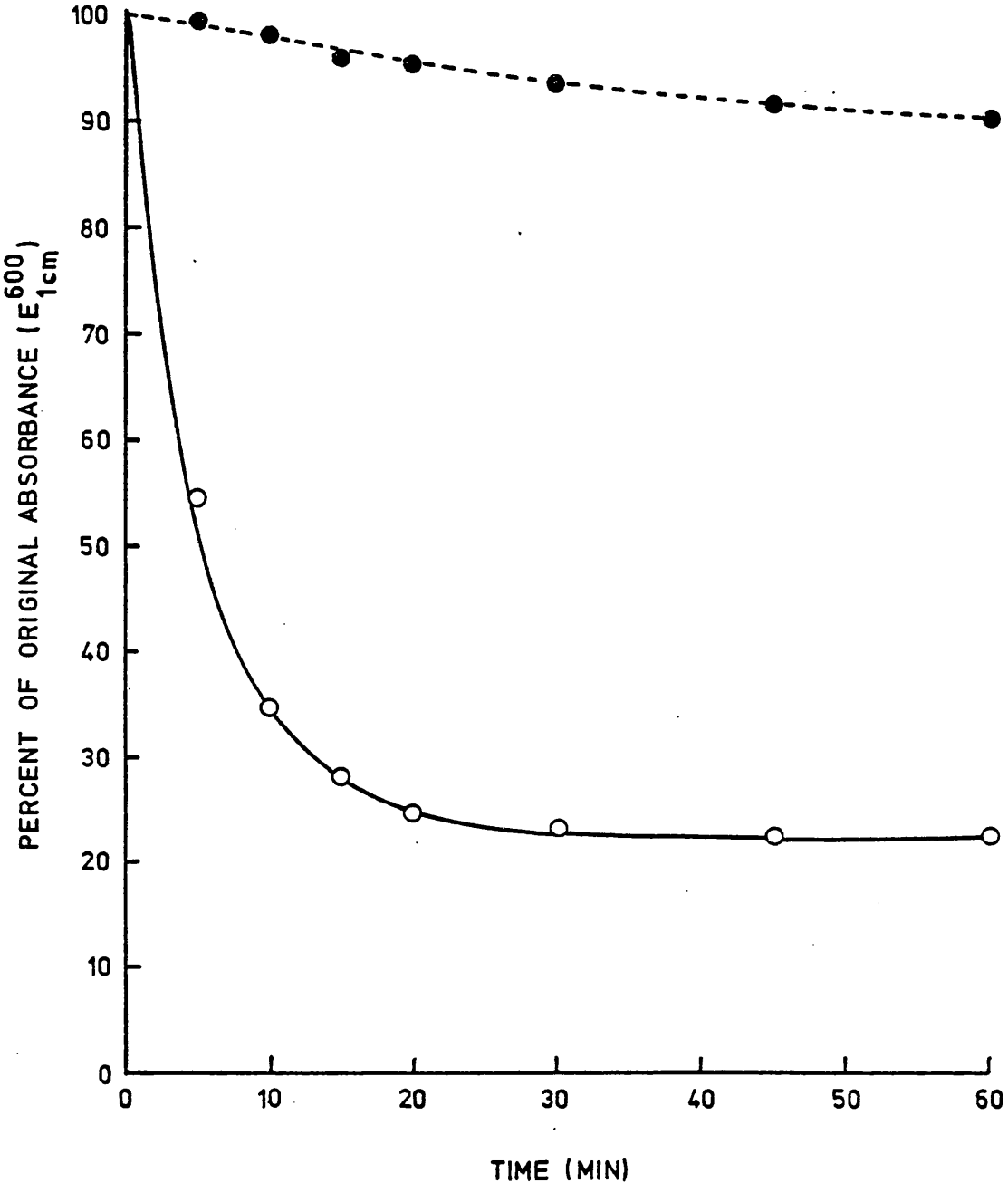


Figure 24. Time-course of the formation of sphaeroplasts from *Saccharomyces cerevisiae* NCYC 366 grown without supplementation, using Zymolyase-5000 (0.4 mg/6 mg dry wt. cells). The dotted line (●) indicates dilution of the suspension (0.1 ml) with buffer (2.9 ml), while the continuous line (○) indicates dilution with water (2.9 ml).

in the suspension were sphaeroplasts (Plate 19). The remaining structures still retained at least 30% of their wall material. A further 30 min incubation (60 min in all) was needed before the suspension contained about 95% sphaeroplasts (Plate 20). The sphaeroplasting process, as a whole, appeared to be a non-synchronous process, with the cells of the population showing differing sensitivities to the enzyme treatment.

Unscarred material was pitted after only 5 min incubation. These pits appeared to start as small holes in the surface and enlarge, with the partially digested material forming bead-like structures at the perimeter (Plate 21). After further digestion the whole of the remaining surface of the wall took on a bead-like appearance (Plate 22). The surface was broken by pits which revealed the invaginated surface of the plasma membrane. In some cases weakening of the cell wall by its conversion to bead-like particles led to splitting of partially digested wall (Plate 23).

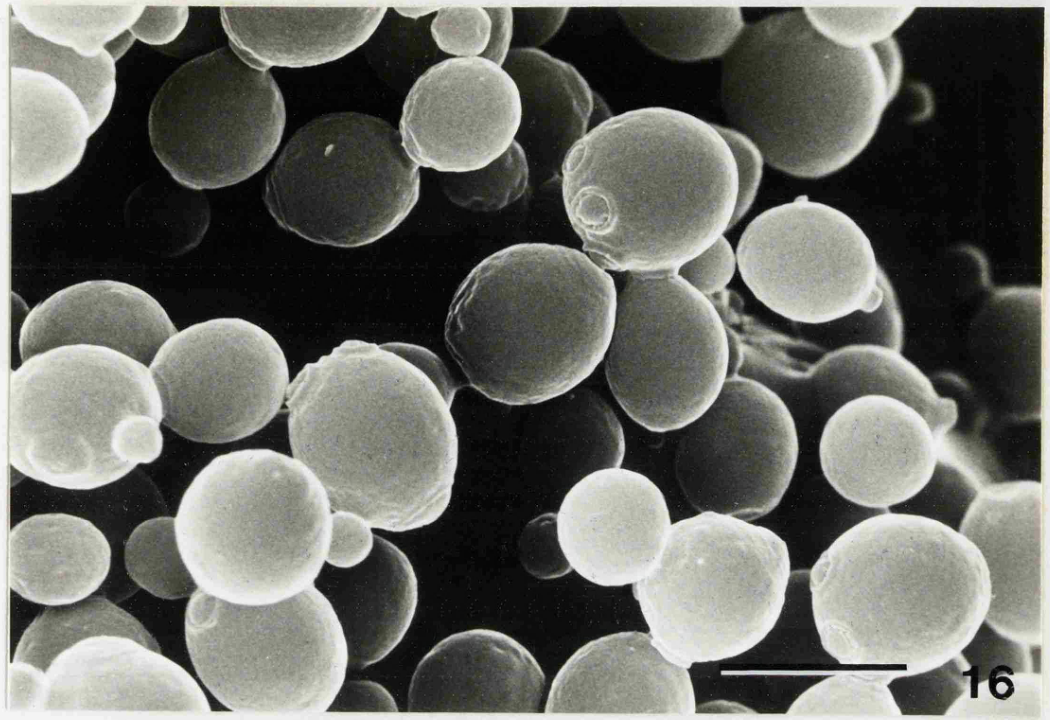
Bud scars, a prominent feature of yeast cells, consisted of a circular raised rim which contained a convex plug that was usually just higher than the raised rim (Plate 24). Both of these structures had small particles attached to them. In most cases surrounding the raised rim there was a less conspicuous outer rim. After 5 to 10 min treatment with Zymolyase the raised rim was decreased in height to below the level of the, now more prominent, outer rim (Plate 25). The convex plug appeared to have been digested leaving a resistant plate at its base since the underlying plasma membrane could not be seen. These partially digested structures, which were seen after 10

min incubation, appeared to be resistant to further digestion as did the material directly surrounding them and were even seen after 60 min of Zymolyase treatment (Plate 26).

The sphaeroplasts produced after 60 min Zymolyase treatment, like PC- or PE-enriched sphaeroplasts, possessed a characteristically invaginated plasma membrane. At high magnification it was possible to discern globular particles (10 to 15 nm in diam.) embedded in the outer monolayer of the plasma membrane, as well as a number of much larger bead-like particles probably of partially digested wall material which were still attached to the plasma membrane (Plate 27). A number of sphaeroplasts possessed depressions on their surface (Plates 20 and 23).

Plate 16. *Saccharomyces cerevisiae* NCYC 366 not enriched in either PC or PE. Bar marker represents 5 μm.

Plate 17. *Saccharomyces cerevisiae* NCYC 366 not enriched in either PC or PE, after 5 min incubation with Zymolyase—5000. The particular material attached to cells is probably partially digested cell wall. Bar marker represents 5 μm.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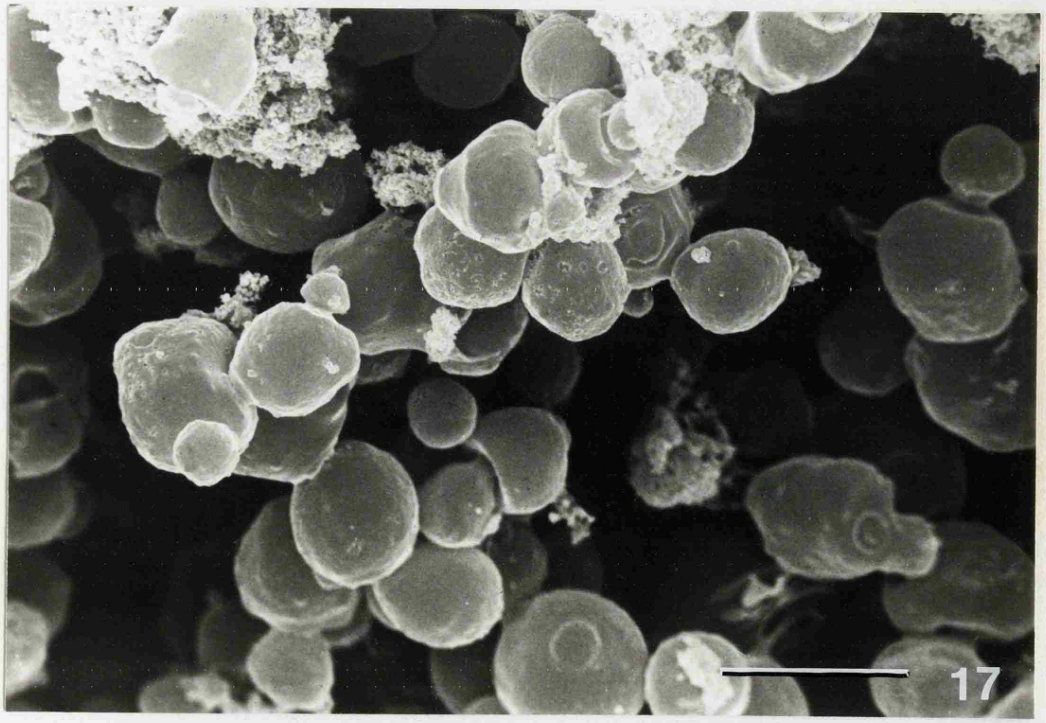
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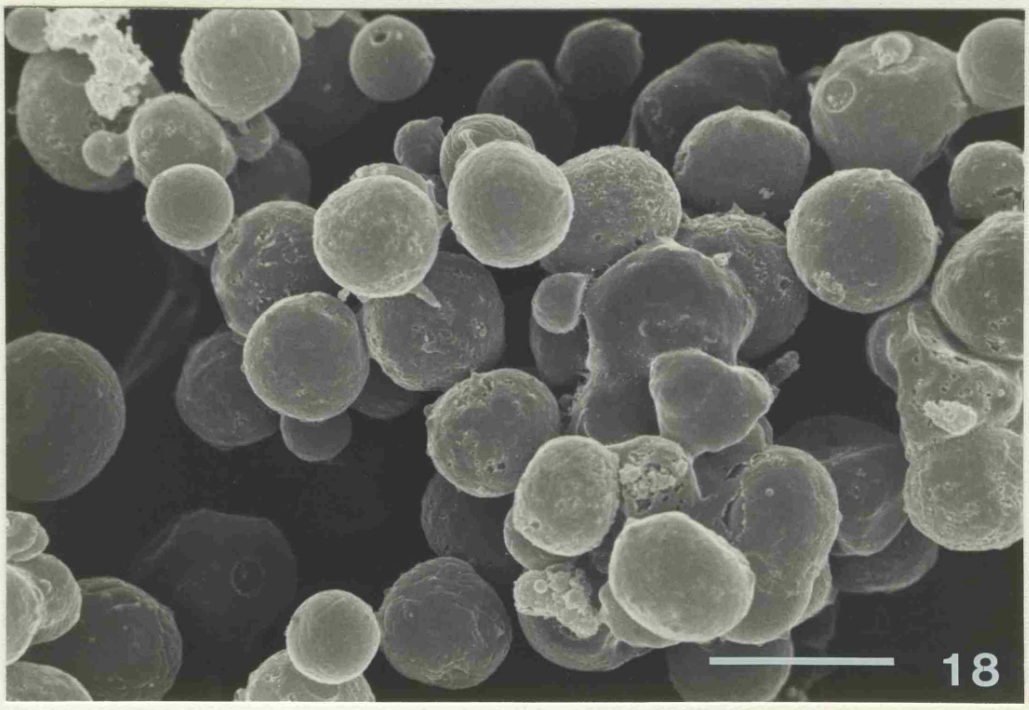
Plate 18. *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE, after 20 min incubation with Zymolyase-5000. About 20% of the cells have been converted to sphaeroplasts. The partially digested cells have a tendency to stick together. Bar marker represents 5 μm.

Plate 19. *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE, after 30 min incubation with Zymolyase-5000. About 25% of the cells have been converted to sphaeroplasts. Bar marker represents 5 μm.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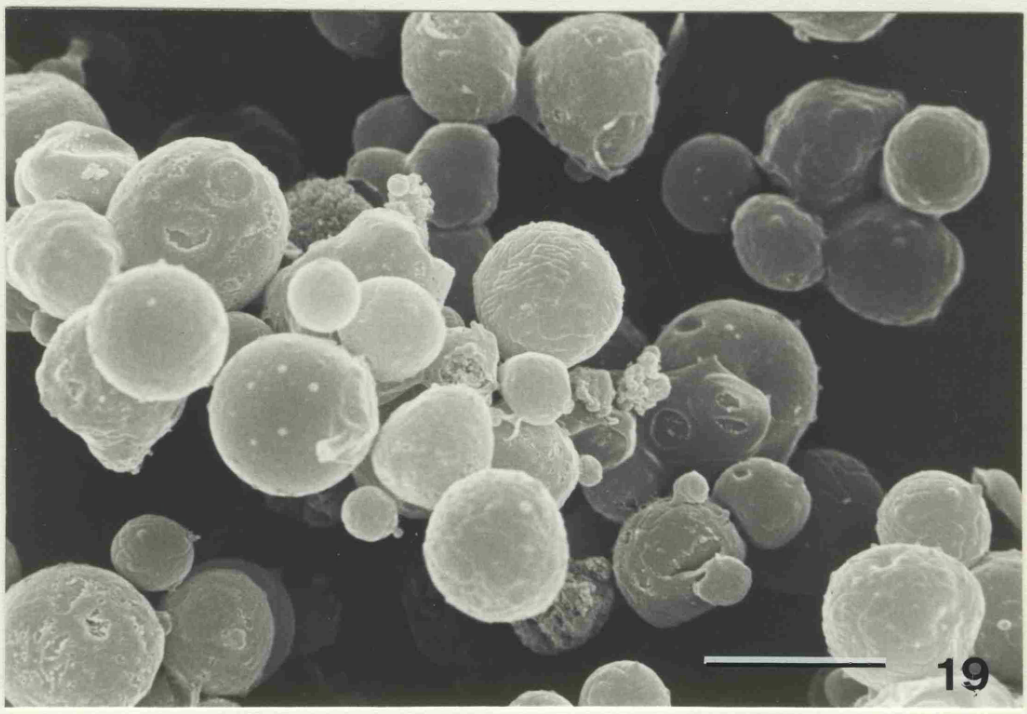
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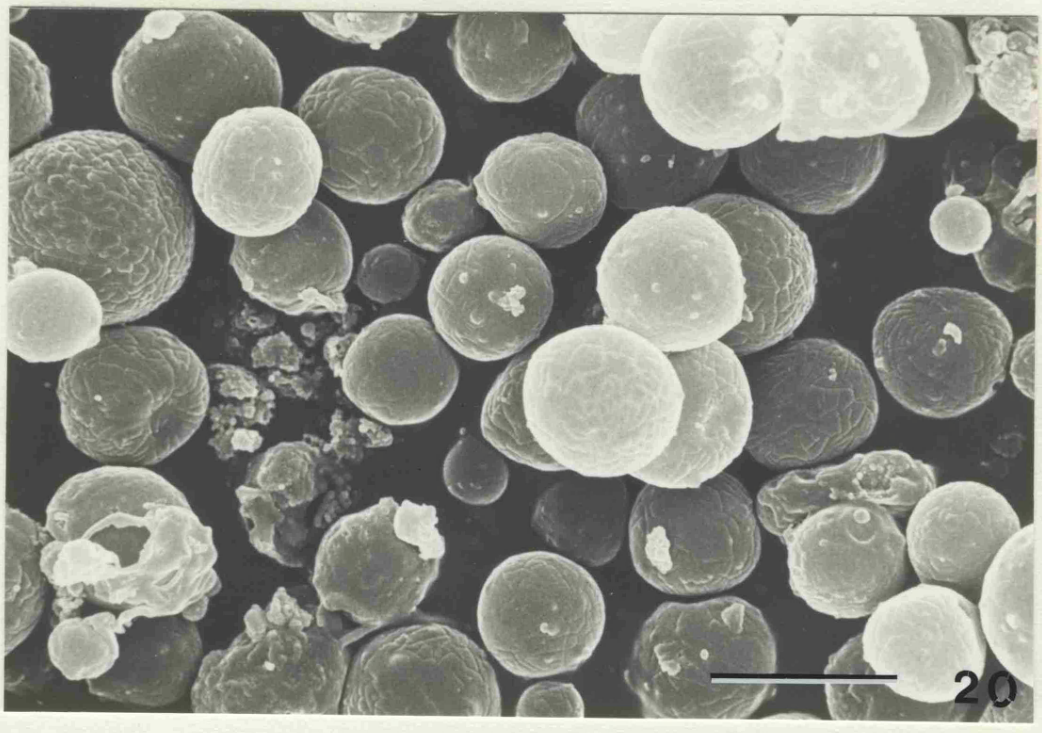
Plate 20. *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE, after 60 min incubation with Zymolyase-5000. About 95% of the cells have been converted to sphaeroplasts. Bar marker represents 5 µm.

Plate 21. Part of cell wall of *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE, after 5 min incubation with Zymolyase-5000. The surface is broken by pits which have at their perimeter bead-like particles of partially digested wall material. Bar marker represents 0.5 µm.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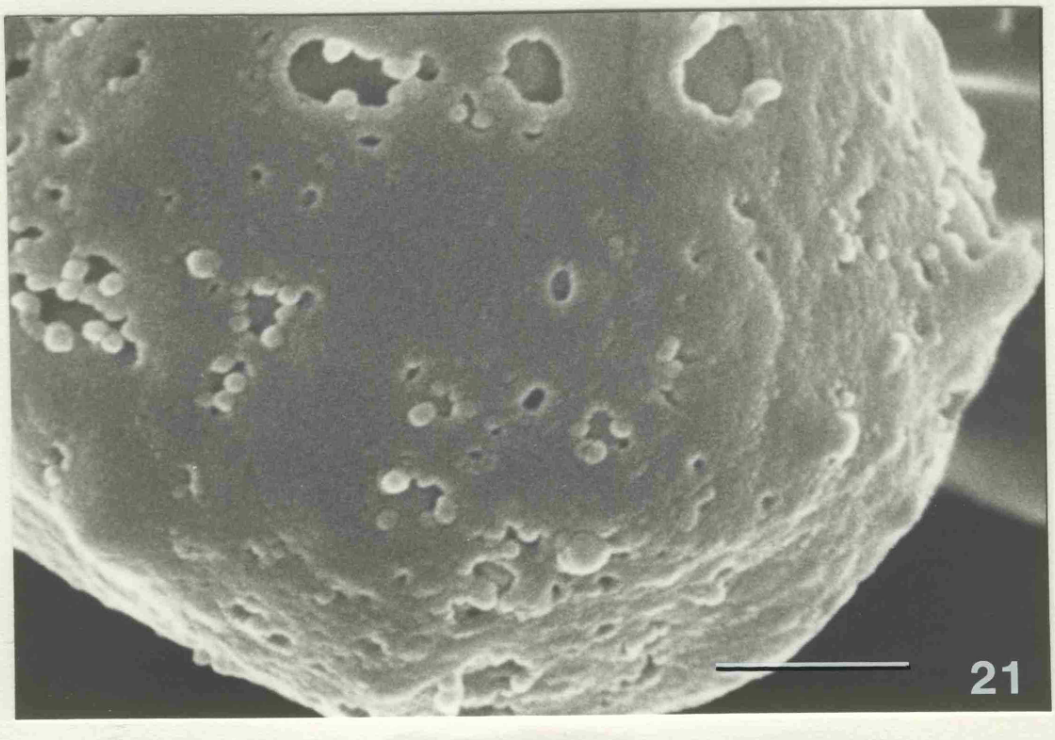
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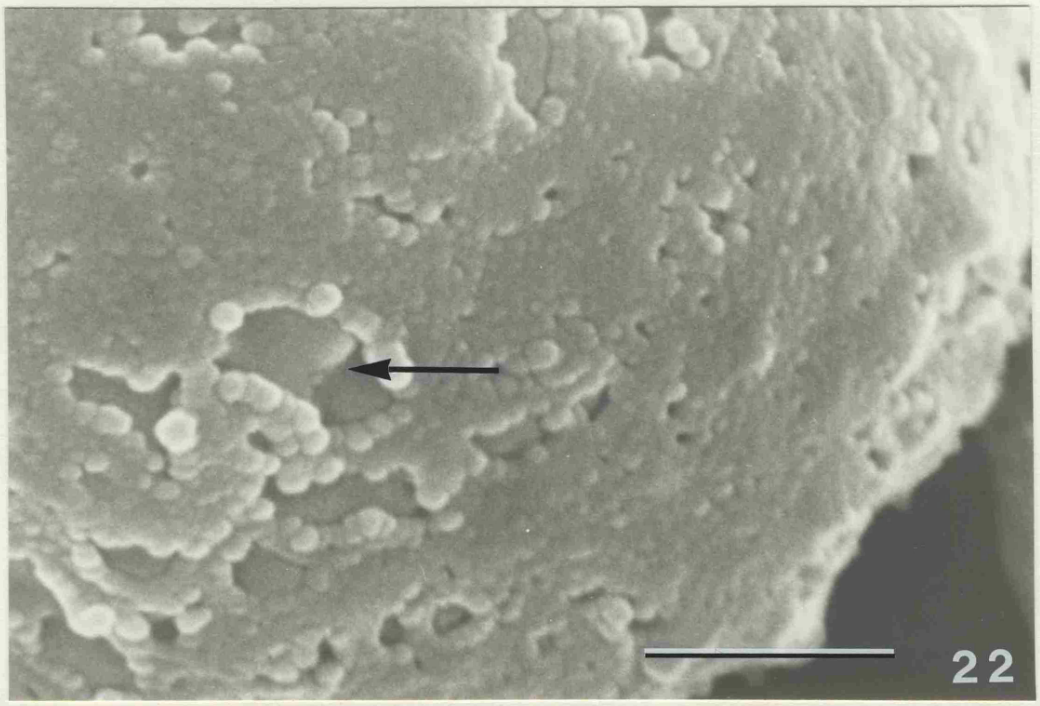
Plate 22. Part of cell wall of *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE, after 30 min incubation with Zymolyase-5000. The cell-wall surface is almost completely converted to bead-like particles. The plasma membrane is visible through fissures in the surface (arrow). Bar marker represents 0.5 µm.

Plate 23. *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE, after 45 min incubation with Zymolyase-5000. The remaining cell wall has a particulate appearance and is apparently splitting. The plasma membrane is characteristically invaginated and retains small particles of cell-wall material (black arrows). The depression (white arrow) in the plasma membrane appears to bear the imprint of a birth scar. Bar marker represents 1 µm.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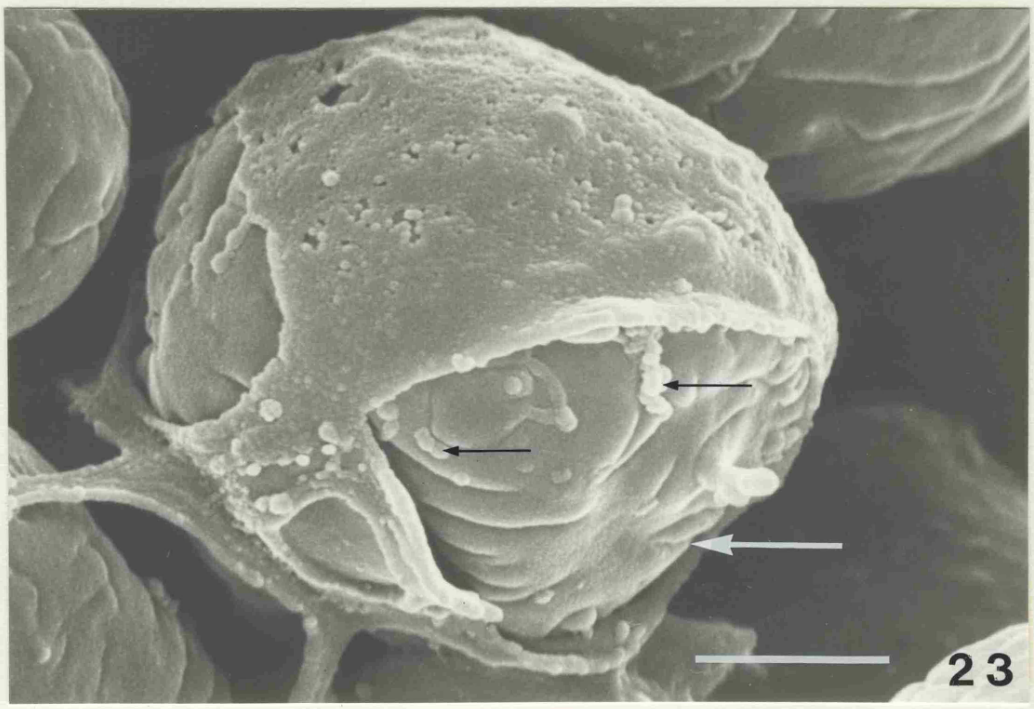
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Plate 24. Bud scars of *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE. The bud scars consist of a raised rim (white arrow) which contains a convex plug, and both of these structures have small particles attached to them. An outer rim is just discernable (black arrows). Bar marker represents 1 μm.

Plate 25. *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE, after 10 min incubation with Zymolyase-5000. The raised rim (white arrow) and plug have been considerably digested. The outer rim (black arrow) appears resistant to Zymolyase treatment as does the material directly surrounding the bud scars. Bar marker represents 1 μm.

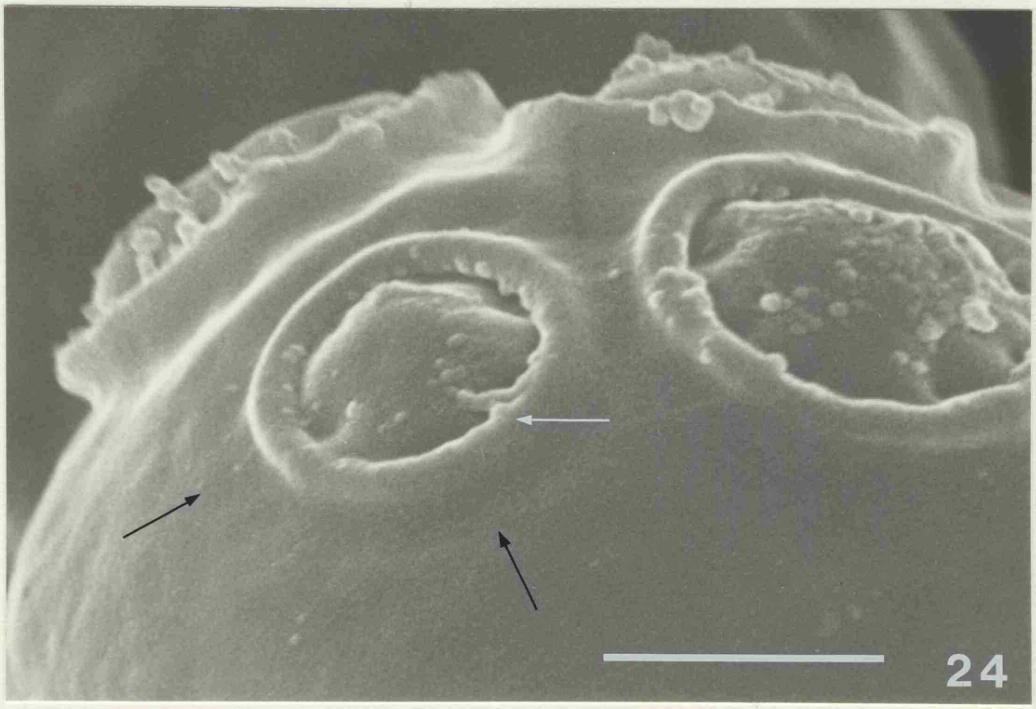
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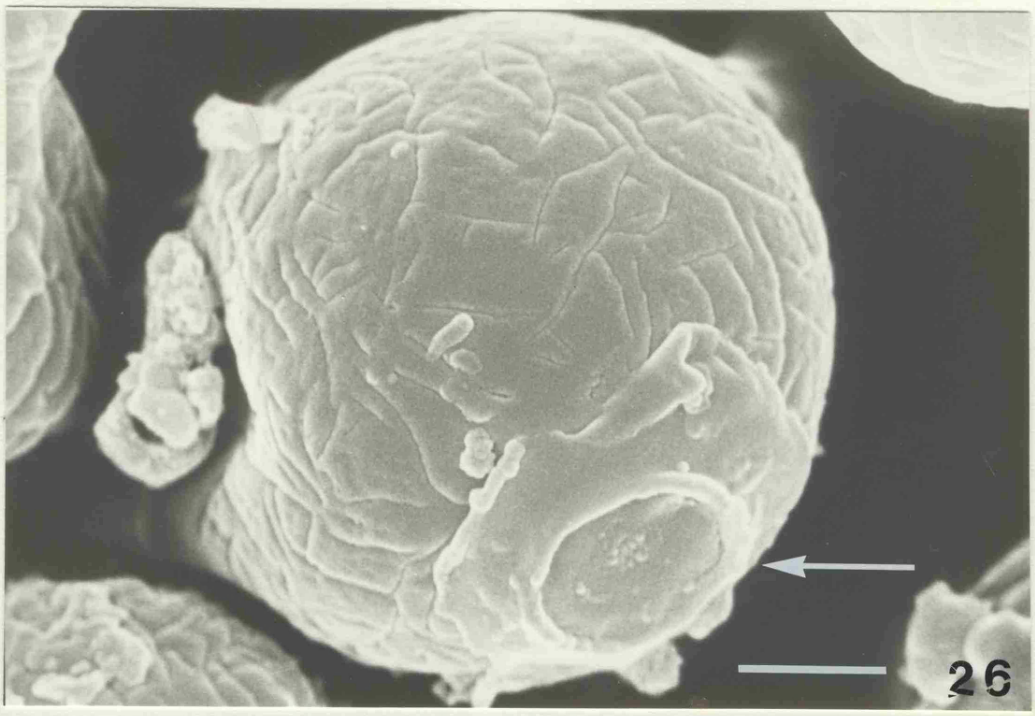
Plate 26. *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE, after 60 min incubation with Zymolyase-5000 . The Zymolyase-resistant substructure of the bud scar (arrow) is attached to the sphaeroplast. Bar marker represents 1 μm.

Plate 27. Part of surface of sphaeroplast of *Saccharomyces cerevisiae* NCYC 366, not enriched in either PC or PE. The plasma membrane is typically invaginated and has globular particles (10 - 15 nm in diam.) embedded in it. The bead-like particle in the centre of the picture is probably partially digested cell-wall material. Bar marker represents 0.25 μm.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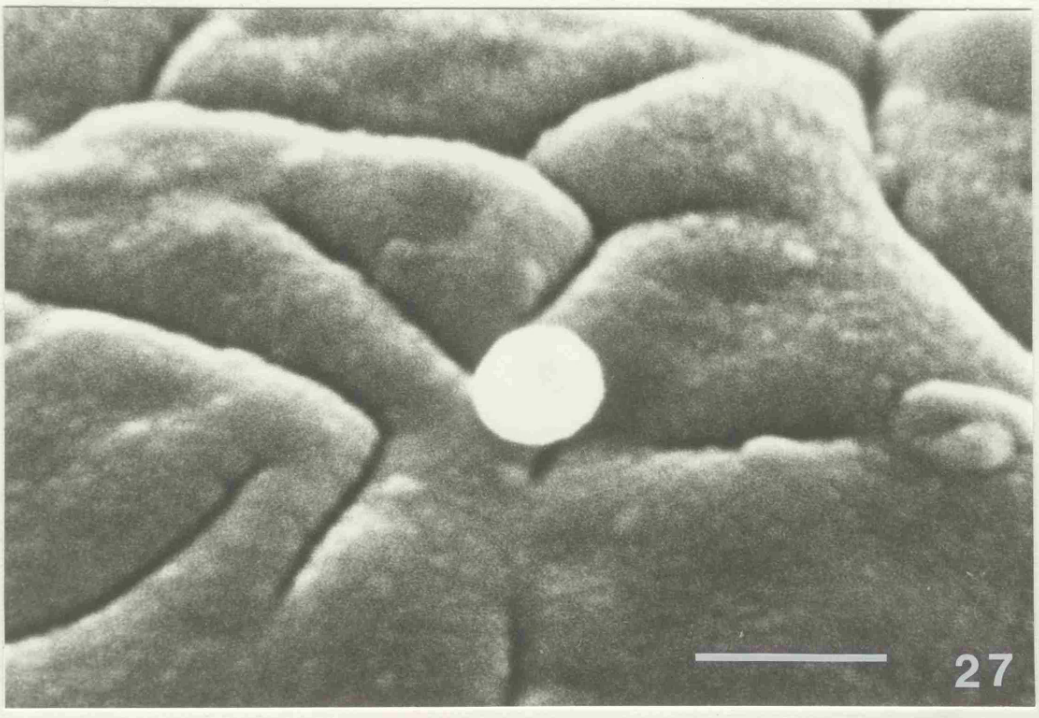
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DISCUSSION

Of the three types of surface-active agents studied, the alkylphenoxy, polyethoxy surfactant, Triton X-100, proved to be the least antimicrobial. The decreased rate of growth which was observed was probably caused by a lowering of the surface tension which led to a decrease in the dissolved oxygen content of the medium and not due to any antimicrobial properties of Triton X-100. This inability to inhibit growth is not surprising since non-ionic surfactants are well known for possessing little or no antimicrobial activity. However, Triton X-100 is known to be a powerful solubilizer of mitochondria and erythrocytes (de Duve,*et al.*, 1962; Weissmann, 1965; Weissmann and Keiser, 1965) and it also proved very effective in solubilizing sphaeroplasts from both types of enriched cells.

One possible explanation for the lack of antimicrobial activity of Triton X-100 is that it does not come into contact with the plasma membrane as it is unable to penetrate the cell wall. Schnaitman (1971) showed that, while Triton X-100 was able to solubilize the plasma membrane of *E. coli*, the outer membrane was unaffected by the surfactant. In an attempt to explain that the different susceptibilities of cells enriched in either PC or PE to SDS were not due to changes in the cell wall, the porosities of the two types of enriched cells to a range of probing molecules were examined. This revealed that the uptake-exclusion threshold of the wall of both types of cells corresponded to a polydisperse polyethylene glycol of number-average molecular weight of 650, which is very close to the value of 620 estimated by Scherrer *et. al.* (1974) for fresh baker's yeast. Since the molecular weight of Triton X-100 has been postulated to be 628 (Inque and Kitagawa,

1976), it is not unreasonable to assume that the surfactant would be excluded from the cell wall.

Although Triton X-100 appeared to be ineffective in preventing growth of *Sacch. cerevisiae* it was able to induce release of cations from cells. If Triton X-100 is unable to penetrate the wall as the previous evidence suggests, it is difficult to understand how it is able to disturb the semipermeable properties of the plasma membrane when it is excluded by the wall. Nevertheless growth in the presence of Triton X-100 was carried out in a K^+-rich nutrient medium, whereas the Triton X-100-induced release of cations was determined in a K^+-free buffer. It may be that any effect on cell permeability caused by Triton X-100 may be reversed by the presence of exogenous K^+. Indeed, the antimicrobial action of polyene antibiotics on yeasts has been reported to be reversed by the presence of exogenous K^+ (Marini *et al.*, 1961). Buffered sorbitol was used to wash and suspend cells for experiments involving release of cations, since washing with water or non-osmotic buffer led to loss of all detectable unbound cations. However, other workers have found that washing fresh baker's yeast with water (Riemersma, 1966a) or *Candida albicans* with a non-osmotic buffer (Gale, 1974) satisfactory for their K^+ release experiments. This evidence suggests that exponentially growing cells of *Sacch. cerevisiae* NCYC 366 release cations very easily, but that release in a K^+ rich nutrient medium does not kill the organisms. However, it is difficult to see how the presence of K^+ in the medium would be able to prevent the disturbance of membrane selectivity by such an effective solubilizing agent as Triton X-100 were it able to reach the membrane, therefore

this phenomenon may have to be explained in another way.

A more plausible reason for the release of cations from cells is that Triton X-100, like most non-ionic surfactants, contains a number of undefined biproducts and unreacted chemicals. Therefore, molecules smaller than Triton X-100 may be able to penetrate the wall and cause release of cations from the cell, but this does not result in permanent membrane damage. Another possible explanation for the Triton X-100-induced release of cations from cells is that active uptake of K^+ and passive leakage out of K^+, which takes place in the periplasmic space and the cell wall, may be disturbed by a lowering of the surface tension around the cells and may cause release of cations without the surfactant coming into contact with the plasma membrane.

Kinsky *et al.* (1969) found that substitution of egg PC for sphingomyelin in sphingomyelin - dicetyl phosphate - cholesterol liposomes caused these structures to become more sensitive to Triton X-100. Furthermore, Inque and Kitagawa (1976) reported that the sensitivity of multilayered and single compartment liposomes, composed of cholesterol and one of a variety of PC derivatives, to Triton X-100 depended on the type of PC used. Kondo (1976) attempted to determine the site of action of non-ionic surfactants on erythrocytes, but was unable to draw a definite conclusion. Nevertheless, he postulated that the lipoprotein portion of the cell membrane was the possible site of non-ionic surfactant action since there have been a number of reports of interactions between this type of surfactant with proteins and lipoproteins (Grundy *et al.*, 1955; Mora *et al.*, 1955; Dowben and Koehler, 1961; Dowben *et al.*,

1961; Koehler and Dowben, 1961). However proteins are unlikely to be the primary site of action since they are known not to be denatured by mild surfactants such as Triton X-100, probably due to their rigid and bulky apolar moieties (Helenius and Simons, 1975). Therefore, like most surfactants, the primary site of action appears to be insertion of the wedge shaped surfactant molecule (Haydon and Taylor, 1963) into the bilayer which causes an increased pressure which induces formation of smaller structures and which results in membrane solubilization (Helenius and Simons, 1975). If adsorption and insertion of Triton X-100 into the bilayer of membranes is the primary site of action, the results presented in this thesis show that the presence of different proportions of PC and PE in the membranes of sphaeroplasts and liposomes appears to have no influence on its action.

The cationic surfactant, CTAB, proved to be the most potent of the three types of surface-active agent investigated, having a considerable effect on growth and viability. The identical electrophoretic mobility curves of cells enriched in PC or PE showed that the wall possesses a net negative charge at pH values between 2 and 9. The potency of the positively charged cationic surfactant might therefore be explained by its attraction for the negatively charged yeast cell.

In studies of binding of CTAB to suspensions of fresh baker's yeast, Fujita and Koga (1966) found that binding of the surfactant was

rapid and almost complete within two minutes, although antimicrobial activity continued for at least one hour. In agreement with this finding,in cells enriched in either PC or PE the antimicrobial activity of CTAB continued over a two hour period. Fujita and Koga (1966) also found that the amount of CTAB needed to kill 100% of the cells in a suspension was ten times that which was needed to cover the surface area of the cell.

Although preliminary experiments with liposomes prepared from commercial phospholipids containing PE proved more sensitive to CTAB than those prepared without PE, this difference did not appear to bear any direct relationship on the effect of the surfactant on cells or sphaeroplasts enriched in PC or PE since they showed no difference in sensitivity to CTAB. The inability to detect K^+ release from liposomes prepared from phospholipids extracted from cells enriched in PC or PE cannot readily be explained. A possible, but not totally convincing, explanation is that due to the number of different phospholipids of which the liposome is composed there is a reconstruction of the liposome at the site of CTAB penetration which enables the liposome to retain its barrier function. Although a similar explanation was postulated by Sunamoto *et al.* (1978) for the action of a functional surfactant on liposomes encapsulating bromothymol blue, these workers were able to detect a certain degree of release of bromothymol blue, whereas no K^+ release was detected from the liposomes prepared from the phospholipid extract of cells. The reason for K^+ release not being detected is probably a complex one caused by the interaction between mixtures of phospholipids, CTAB and K^+ which is not readily explained.

Many workers have postulated that the site of action of cationic surfactants is phospholipid (Gilby and Few, 1960; Kondo and Tomizawa, 1966; Riemersma, 1966b). Kondo (1976) suggested that surface-active cations that reach the phospholipid portion of lipoproteins will interact electrostatically with the phospholipid molecule and replace some of the phospholipid molecules, causing them to move out of the plasma membrane. This replacement of phospholipids with surface-active cations may result in the partial disruption and/or alteration in membrane permeability. Cartledge *et al.* (1977) showed, through pH-electrophoretic mobility studies, that the plasma membrane of *Sacch. cerevisiae* NCYC 366 carries a net negative charge at pH values above 3. Therefore in experiments reported in this thesis the plasma membrane would carry a negative charge, and therefore would electrostatically attract CTAB. The net negative charge would probably be contributed by ionization of phosphodiester groups on phospholipids and carboxyl groups on acidic - amino acid residues of proteins (Somers and Fisher, 1967). If electrostatic attraction between the plasma membrane and cationic surface-active agents is the primary interaction, then both phospholipid and protein would be involved. Both PC and PE are zwitterionic at the pH values used in this study (Papahadjopoulos and Miller, 1967). Therefore since both phospholipids possess a similar phosphodiester group any differences in interaction with CTAB would be caused by repulsion due to the positively charged quaternary ammonium group of PC and the primary amine group of PE. Since differences in the ionization of these positively charged groups are unlikely to influence the net surface charge of the membrane at the degree of enrichment involved, the action of CTAB would not be expected to be affected by enrichment of plasma membranes with PC or PE.

The mode of action of surface-active agents is now generally accepted to involve adsorption of the monomer onto the membrane surface and penetration of the bilayer (Reman *et al.*, 1969; Helenius and Simons, 1975). However in the past, both proteins and lipids have been implicated by various workers as the sites of action of the anionic surfactant, SDS. Gilby and Few (1960), who studied lysis of protoplasts from *Micrococcus lysodeikticus*, concluded that SDS interacted with membrane proteins or lipoprotein complexes. From studies carried out on the haemolytic action of a variety of surface-active agents, Kondo and Tomizawa (1966) proposed that lysis was brought about by co-operation of surfactants with cholesterol and proteins as had been previously proposed by Pethica and Schulman (1953) and Ross and Silverstein (1954), respectively. Phospholipids were implicated as the site of action of SDS by Gilby and Few (1957) from studies of lysis of protoplasts of *Micrococcus lysodeikticus*. Ten years later, from solubilization studies of the plasma membrane of *Mycoplasma laidlawii*, Engleman *et al.* (1967) concluded that the principal site of action of SDS was on the lipid component rather than the protein component of the membrane. More recently, Kondo (1976) proposed that, in the lysis of erythrocytes at pH 7.4, the principal site of action of anionic surfactants was the protein component, but at lower pH values both proteins and phospholipids were important in surfactant action.

There were no apparent differences in the ultrastructure between the two types of enriched cells before or after treatment with SDS. Unlike *E. coli*, where one of the first effects is to solubilize the plasma membrane (Woldringh, 1970; Woldringh and van Iterson, 1972), after treatment with SDS in both types of enriched cell the plasma membranes appeared intact. However, the characteristic invaginations of the plasma membrane which retain their structure after sphaeroplast formation, were absent from the membranes of treated cells. This suggests that SDS causes structural alteration of the plasma membrane. There is also an indication that SDS passes through the plasma membrane and into the cytoplasm since the cell vacuole appeared disrupted and the vesicles and mitochondria were absent from SDS-treated cells. Although these are interesting observations, disruption of the cell vacuole and solubilization of the cytoplasmic inclusions are almost certainly secondary effects, the primary lesion being located at the plasma membrane.

Since SDS is apparently able to penetrate the plasma membrane, the greater loss of viability of, and cation release from cells with plasma membranes enriched with PE induced by this surfactant may be explained by the preferred orientations of the polar head-groups of the phospholipids in the outer monolayer on the plasma membrane. Using X-ray diffraction (Hitchcock *et al.*, 1974) and infrared dichroism (Akutsu *et al.*, 1975), the ethanolamine dipole has been found to be orientated parallel to the surface of the membrane at temperatures below the phase transition (Fig. 25). The same orientation has been inferred from ^{31}P and deuterium nuclear magnetic resonance measurements

of bilayers of dipalmitoyl-3-*sn*-phosphatidylethanolamine above the phase-transition temperature (Seelig and Gally, 1976), with the ethanolamine group rotated flat on the surface of the bilayer. By extrapolation the X-ray long spacings of PC and PE fatty-acyl homologues to zero hydrocarbon chain length, Phillips *et al.* (1972) concluded that the preferred orientations of the choline and ethanolamine dipoles are perpendicular and parallel, respectively, to the bilayer surface (Fig. 25). These workers concluded that this conformation would make the choline head-group more hydratable than the ethanolamine head-group, since the energy of such a system would be lowered by the presence of water molecules hydrogen bound between the anionic oxygen atoms of the phosphate groups. Indeed, other workers have found, using differential-scanning calorimetry (Ladbrooke and Chapman, 1969) and hydration studies (Jendrasiak and Hasty, 1974), that pure PC adsorbs more water than pure PE. Seufert (1973) showed that penetration of SDS into the outer monolayer of lipid bilayers was necessary to induce cation-permselectivity. Therefore, if PE exists in a less hydrated form than PC in the outer monolayer of the plasma membrane, this would possibly render cells with plasma membranes enriched in PE more susceptible to penetration by the hydrophobic chain of SDS.

Recently the perpendicular orientation of the choline head-group has been disputed by Seelig *et al.* (1977) who, using ^{31}P and deuterium nuclear magnetic resonance of unsonicated PC bilayers in the liquid crystalline state, concluded that the choline head-group lies parallel to the bilayer. Further work by Jendrasiak and Mendible (1976) showed,

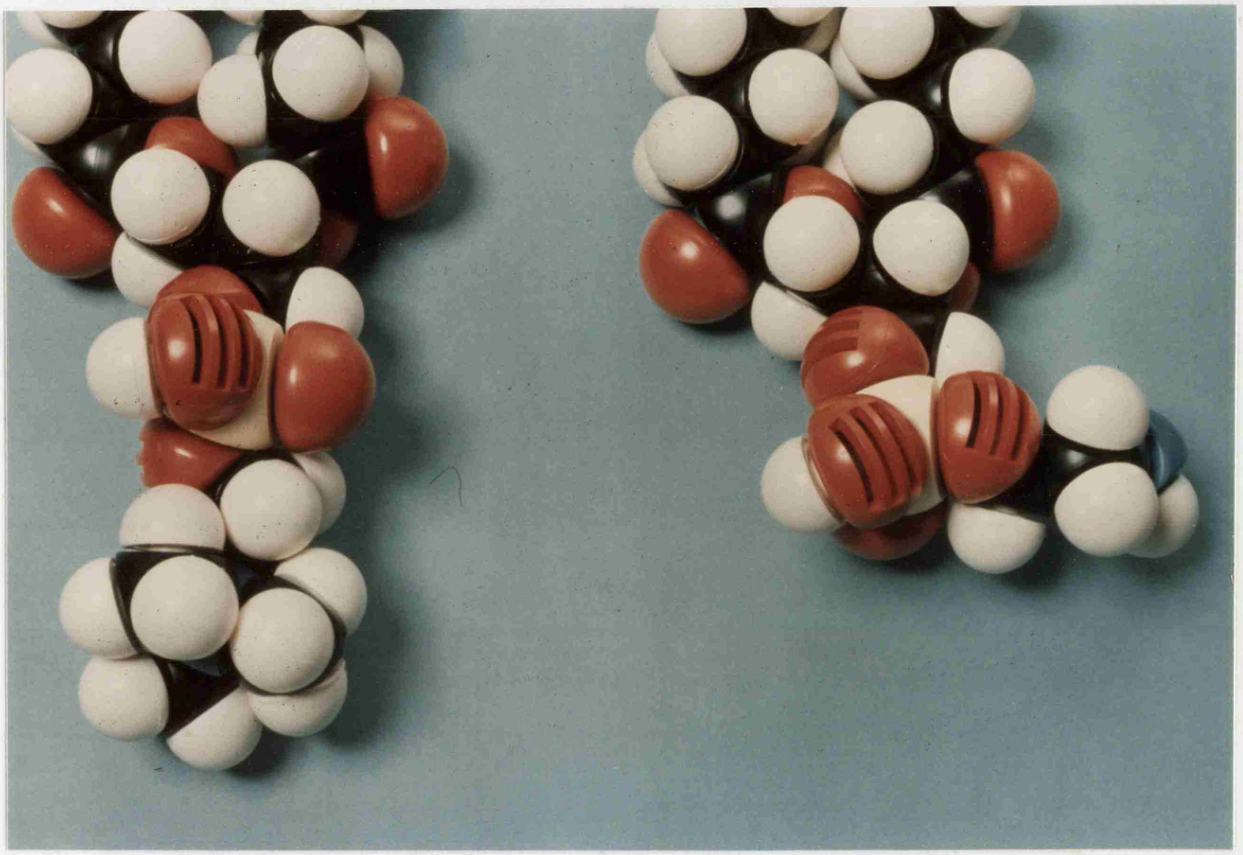

Figure 25. Space-filling models of the head-groups of phosphatidylcholine (left) and phosphatidylethanolamine (right).

in hydration studies of mixtures of PC and PE, that PE was as hydratable as PC. This discovery of a difference between the hydration capacity of pure phospholipids as compared with phospholipid mixtures led these workers to conclude that the perpendicular orientation of the PC head-group prevented the PE head-group from lying parallel to the surface of the bilayer. In multilayered liposomes, prepared from phospholipids extracted from either type of enriched cells, it is not unreasonable to assume that PC and PE would be more or less randomly distributed in the plane of the membrane. Therefore, in these structures, PE would probably be as hydrated as PC and so it is not surprising to find that there are no differences in the SDS-induced release of K^+ from these structures. Although the conformation of the PC and PE head-groups is still open to speculation, it appears that pure PE is less hydrated than pure PC. Thus, if the greater susceptibility of PE-enriched cells to SDS can be explained by the less hydrated nature of the PE head-group, then PE would have to exist in isolated patches or slicks in the other monolayer of the plasma membrane. This type of non-random distribution in the plane of the bilayer could be referred to as mosaicism.

Although this type of non-random distribution in the plane of the membrane has yet to be reported in *Sacch. cerevisiae*, it is inferred by a number of factors. One of these is that scanning electron micrographs of sphaeroplasts revealed characteristic invaginations in the plasma membrane which have been previously seen in freeze-etch electron micrographs of other workers (Moor and Mühlethaler, 1963; Streiblová, 1968; Matile *et al.*, 1969; Nečas *et al.*, 1969; Takeo *et al.*, 1976).

The fact that these invaginations are maintained during and after removal of the wall indicates that there is a high degree of structural organisation in the plasma membrane. In agreement with Takeo *et al.* (1976), sphaeroplasts from (larger) older cells possessed deeper and more frequent invaginations. Although the function of these invaginations in *Sacch. cerevisiae* is not known, Hereward (1976) suggested that they were the sites of production of wall fibrils in *Schizosaccharomyces pombe*. However, since there are no apparent changes in the invaginations during or after sphaeroplast formation in *Sacch. cerevisiae* NCYC 366, the difference in sensitivity to SDS between cells and sphaeroplasts enriched in PC or PE will need another explanation.

Since buds appear mainly at the opposite end of the cell to the birth scar (Barton, 1950; Nickerson, 1963), this suggests that the underlying membrane has a specificity which dictates where a new bud will be formed on the cell. This hypothesis, concerning topographical specificity in the yeast plasma membrane, is further supported by the fact that the bud scars are often the last parts of the wall to be removed, which suggests that there is a stronger attachment between this part of the wall than other parts (Plate 26). The globular particles (10 to 15 nm in diam.) that were observed embedded in the outer monolayer of the plasma membrane (Plate 27) were similar to those observed in *Sacch. cerevisiae* by other workers (Moor and Mühlethaler, 1963; Streiblová, 1968; Matile *et al.*,1969; Nečas *et al.*, 1969; Takeo *et al.*, 1976). Matile *et al.* (1967) reported that these particles consisted of mannan and protein in the ratio of 5:2, but had no enzymatic activity and from this they concluded that these particles were precursor units of the

cell wall. The hexagonal arrangements of these globular particles has also been observed by the aforementioned workers, although Takeo *et al.* (1976) who studied exponential- and stationary-phase cells, concluded that these arrangements were only present in stationary-phase cells and indicated parts of the membrane that were not in contact with the cell wall. No such arrangements were seen in the membrane of sphaeroplasts from the two types of enriched cells, which suggests that there is a firm attachment of the plasma membrane with the cell wall. This attachment was confirmed in the study of sphaeroplast formation where it was apparent that portions of partially digested wall material remained on many of the cells after the bulk of the wall material had been removed (Plates 19, 20 and 27). It is not unreasonable to assume, considering the prominence of the globular particles on the plasma membrane surface, that at least some of these form a linkage between the wall and the membrane.

Another type of linkage between wall and membrane has recently been postulated following the discovery in bacteria of phosphatidyl-kojibiosyl diglyceride in the membrane, which is bound covalently through a phosphodiester bond to the polyglycerol phosphate moiety of lipo-teichoic acid which could span the wall and membrane (Pieringer and Ganfield, 1975). Brennan and Lösel (1978) suggested that in yeasts some glycerophosphosphingolipids may be associated with mannan, which would similarly anchor these lipids in the membrane. Therefore, if movement of lipids in certain parts of the plasma membrane is restricted by linkage of lipids (and possibly proteins), to the wall, this may enable slicks of phospholipid (possibly PE) to exist in the outer

monolayer as a result of steric hinderance. Following removal of the wall during sphaeroplast formation, this ordered structure of the plasma membrane, as dictated by the wall, would be lost and any slicks of phospholipid would become dispersed giving rise to a more random distribution of phospholipids in the plasma membrane. Thus, if slicks of less hydrated PE molecules exist in the outer monolayer of the plasma membrane, they would be dispersed and the PE molecules would probably become as hydrated as PC, which would explain why PE-enriched sphaeroplasts were not as susceptible to SDS as cells. However, this does not readily explain why PC-enriched sphaeroplasts are more susceptible than those enriched in PE.

Another type of non-random distribution which has been reported in biological membranes is the asymmetric distribution of phospholipid molecules between the monolayers of the bilayer. Bretscher (1972) was first to postulate that the majority of the PE residues were present in the inner half of the bilayer after finding that more was labelled when human erythrocyte ghosts, rather than intact erythrocytes, were exposed to ^{35}P formylmethionyl methyl phosphate. This asymmetric distribution was later confirmed by Zwaal *et al.* (1973) and Verkliej *et al.* (1973) using specific phospholipases,and Gordesky and Marinetti (1973), using 2,4,6-trinitrobenzenesulfonate who found that the outer monolayer of the erythrocyte consists predominantly of PC and sphingomyelin, and that PE and phosphatidylserine are localized to the interior of the monolayer. Similar asymmetric distributions of phospholipids have been subsequently reported in the membranes of milk fat globules (Patton and Keenan, 1975), and influenza virions

(Tsia and Lenard, 1975; Rothman *et al.*, 1976) which led Tsia and Lenard (1975) to suggest that this pattern of asymmetric phospholipid distribution may be a general feature of plasma-membrane structure.

However more recently Nilsson and Dallner (1977) reported that, in the subcellular membranes of rat liver, PE and phosphatidylserine were present in the outer monolayer, phosphatidylinositol, sphingomyelin and cardiolipin on the inner monolayer, while PC was evenly distributed between the bilayers of the membranes. Barsukov *et al.* (1976) were the first to report asymmetric distribution of phospholipids in the plasma membrane of bacteria using *Micrococcus lysodeikticus*. Subsequently, Rothman and Kennedy (1977) have reported that, in *Bacillus megaterium,* 33% of the PE was located in the outer monolayer and Bishop *et al.* (1977) have claimed that 60% of the PE in the plasma membrane was located in the outer monolayer. Recently Paton *et al.* (1978) have reported that 70% of the total phospholipid exists on the outer half of the bilayer of the plasma membrane of *B. amyloliquefaciens* with PE being predominantly present on this half of the bilayer. It is therefore very likely, considering the weight of the evidence from the membranes of other organisms studied, that an asymmetric distribution of phospholipids exists in the plasma membrane of *Sacch.cerevisiae*, but it is not possible to predict with any certainty which phospholipids would be predominant on either side of the membrane. If PC is predominant on the outside and PE predominant on the inside of the plasma membrane of *Sacch. cerevisiae,* as the other eukaryotic membranes studied,this may explain why there is a difference in susceptibility between cells enriched in PC or PE.

Cells with plasma membranes enriched in PC could be predicted to have little PE in the outer monolayer due to the preference of increased proportions of PC for this location, and therefore these membranes would possess fewer, smaller slicks of PE which would make these cells less sensitive to SDS. It is difficult to explain why PC-enriched sphaeroplasts are more sensitive to SDS using asymmetric distribution. However the equal distribution of phospholipids between the bilayers of multilayered liposomes extracted from cells enriched in PC or PE would explain why no differences in sensitivity to SDS were observed in these structures. Although the effect of asymmetric distribution of phospholipids on the sensitivity of the two types of enriched cells is at the present time only speculation, it is a potentially significant factor which will only be better understood when more information is available on the plasma membrane of *Sacch. cerevisiae*.

It is conceivable that enrichment of the plasma membrane of *Sacch. cerevisiae* with PC or PE might affect the activity of membrane-bound enzymes that catalyse synthesis of cell-wall components and this may explain the different sensitivities of the two types of enriched cells to SDS. However, my data did not reveal any differences in the properties of walls of cells enriched in either phospholipid. The most compelling evidence was the identical porosity profiles of cells enriched in PC or PE, which show that SDS (molecular weight 288) should be able to penetrate walls of both types of enriched cells with equal facility. Nevertheless, as the age of the culture increased, so the sensitivity to SDS decreased, although the PE-enriched cells were still more sensitive than PC-enriched cells. The diminished sensitivity to SDS

of these older cells is undoubtedly due to changes in the wall, since cultures harvested after 36 h were resistant to β-glucanase.

In conclusion the fact that only the action of SDS is affected by enrichment of the plasma membrane with PC or PE indicates that there is a difference in the mode of action between this surface-active agent and the other two types of surfactant used in this study. This difference in action of SDS and other anionic surfactants has been reported by various other workers (Gilby and Few, 1960; Kondo and Tomizawa, 1966; Kondo, 1976) although they all implicate proteins or lipoprotein complexes as being the primary site of action. Despite the mechanism by which SDS acts on membranes not being fully understood, it appears that phospholipids are important in the interaction of this surfactant with the plasma membrane. It may be that the difference in interaction of the negatively charged surfactant with membranes enriched in PC or PE is due to the different electrostatic interactions between the positively charged choline or ethanolamine amino groups coupled with the different hydratabilities of the polar head-groups.

Although electron microscope studies were initially intended to yield information which would explain the different susceptibilities of PC-or PE-enriched cells and sphaeroplasts to SDS, they also revealed a great deal of information concerning the structure of *Sacch. cerevisiae*

and the process of sphaeroplast formation.

It is clear that not all the cells in a population have the same wall structure or composition since some appear more susceptible to β-glucanase than others. This results in sphaeroplast formation being non-synchronous with maximum osmotic sensitivity not coinciding with completion of this process. In agreement with Streiblová (1968), the study showed that osmotic fragility is not a reliable criterion for establishing the completion of the sphaeroplast formation.

Zymolyase-5000 is prepared from filtrates of cultures of *Arthrobacter luteus* (Kitamura *et al.*, 1971) and has been shown to be free from β-(1→2), β-(1→4) and β-(1→6) glucanase and phosphomannanase activities. It contains endo-β-(1→3) glucanase activity which is active on some but not all β-(1→3) glucans with a preference for glucans possessing a firm molecular aggregation due to hydrogen bonding (Kitamura and Yamamoto, 1972). Therefore, the rapidly assumed bead-like appearance of the unscarred wall material during digestion suggests that the β-(1→3) glucans hydrolysed by Zymolyase are not uniformly accessible to the enzyme. This finding suggests that in *Sacch. cerevisiae* NCYC 366 β-(1→3) glucans may not be located in an inner fibriller layer which is covered by an amorphous shell of β-(1→6) glucan, as suggested by Kopecká *et al.* (1974). The sticking together of sphaeroplasts of partially digested cells may be caused by a release of mannan (yeast gum) which as well as being located at the surface of the cell wall may also be enmeshed with the glucans in deeper layers of the wall (Ballou, 1974; Cabib, 1975).

The most common type of sphaeroplast formation is emergence of the structure through a hole that has been digested in the cell wall (Villanueva, 1966). However with *Sacch. cerevisiae* NCYC 366, it appears that the unscarred material is equally digested into bead-like particles which are eventually sloughed off or totally digested. Both types of sphaeroplast formation have previously been observed (Villanueva, 1966) although it is not surprising to encounter the less common formation with *Sacch. cerevisiae* NCYC 366 in view of the marked susceptibility of exponentially growing cells to β-glucanase.

The plug and part of the raised rim of the bud scar were digested by Zymolyase to reveal a resistant substructure which was similar to those seen by Cabib and Bowers (1971). These workers reported that this substructure was composed of chitin which is consistent with its resistance to Zymolyase-5000 which is free from detectable chitinase activity (Kitamura and Yamamoto, 1972). Cabib and Bowers (1971) also postulated that the resistant substructure of the bud scar was the remains of the primary septum which is formed during separation of a daughter cell. Consequently the wall material overlying the resistant substructure must be made up of glucans and mannan as suggested by other workers (Cabib and Bowers, 1971; Bauer *et al.*, 1972; Bush and Horisberger, 1973). The β-glucanase-resistant outer rim, which was often seen in this study has not been reported before. It may be the perimeter of the region of cell-wall growth during bud formation. This structure may be unique to *Sacch. cerevisiae* NCYC 366 and it is possibly due to the unusual nature of the cell wall of this strain.

A conspicuous feature of populations of sphaeroplasts was the number of sphaeroplasts with a depression in the surface. While these could conceivably be artifacts of fixation and drying, they appeared to correspond to the site where a birth scar had been on the original cell as some bore, what appeared to be, the imprint of a birth scar (Plate 23). Because of the site of the depressions and because only one was usually seen on a sphaeroplast they probably correspond to birth scars rather than bud scars. Since sphaeroplasts have the ability to maintain depressions and invaginations after the removal of the cell wall, this is further evidence for the existence of a cytoskeleton in *Sacch. cerevisiae* which was postulated by Santos *et al.* (1978) after finding tubulin-like proteins in the plasma membrane.

Scanning and transmission electron microscopy revealed a number of vesicles protruding just beneath the surface of the plasma membrane (Plates 9 and 14), a phenomenon that has also been observed by Santos *et al.* (1978). This finding may explain why Hossack (1975), after lactoperoxidase-catalysed iodination of sphaeroplasts of *Sacch. cerevisiae* NCYC 366 and separation of sphaeroplast components on a sucrose gradient, found a high degree of labelling in the vesicle fraction. Although lactoperoxidase-catalysed iodination is only supposed to label proteins on the exterior of the plasma membrane, these results indicate that a large proportion of vesicles are also labelled. This suggests that ^{125}I may be able to penetrate the plasma membrane at points of vesicle protrusion and label them.

Cartledge *et al.* (1977) isolated two sizes of vesicle (0.62 μm and 0.43 μm) from *Sacch. cerevisiae* NCYC 366, and because the larger vesicles are known to fragment into smaller ones (Sentandreu and Northcote, 1969; Wiemken *et al.*,1970) these workers speculated that the smaller ones may contribute to growth of the plasma membrane and cell wall. Therefore removal of the cell wall may trigger the migration of vesicles to the plasma membrane in order to contribute to synthesis of new wall material. Indeed in preliminary attempts to regenerate cell-wall material on sphaeroplasts in a solid nutrient-medium, the vesicles became even more prominent on the surface and appeared to be the principal sites of new cell-wall material. A similar phenomenon has also been observed in zoospores of *Phytopthtara palmivora*, where the migration of vesicles to the plasma membrane was coincident with the formation of cell-wall material (Bartnicki-Garcia, 1973).

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